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Development of Cycling Probe Technology Assay for Microfluidics-Enabled Platform *(Final Report)*

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FINAL REPORT FOR PROJECT ENTITLED:

Development of Cycling Probe Technology Assay for Microfluidics-Enabled Platform.

Contract No. R646/001/EDM

(Period from April 1, 1997 to March 31, 1999)

Submitted to: Science and Professional Services
Western Region, Public Works and
Government Services Canada

and

Hazard Assessment Section,
Defence Research Establishment Suffield

Submitted by: T. Dickinson Laing, RTM
James Kelly Pon, RTM
M. Mah, QAO
D. Mah, Projects Director

CWBios Inc.
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Executive Summary

We have investigated the utility of adapting a cycling probe technology (CPT) assay to a microfluidics format for the identification of the biological warfare simulant *Erwinia herbicola*. We designed and screened a series of CPT probes for *Er. herbicola*, 5'-labelled with either fluorescein or Cy-5 and 3' labelled with biotin, to facilitate detection by capillary electrophoresis with laser-induced fluorescence (CE-LIF). Two of nine probes screened (EH4 and EH8), based on the beta-glucosidase and the tyrosine phenol lyase genes, were found to recognize genomic DNA from *Er. herbicola*, but were not specific for that organism. EH8 probe was used as a test probe to validate the assay on a benchtop capillary electrophoresis unit, with a final goal to adapt the assay to a microchip format. The lower detection limit of the assay with CE-LIF detection for purified genomic DNA was 10^5 chromosomal copies. The assay lent itself to adaptation to CE-LIF detection, but with some significant sensitivity and specificity issues. Although absolute quantitation of genomic target was not achieved with this assay, it was able to determine relative estimations of target in pure samples. This assay has not yet been tested with field trial samples. In summary, we have been able to adapt this assay to a rapid CE-LIF detection format suitable for microchip use with a detection time of approximately 5 minutes.

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Abbreviations

BGL	beta-glucosidase
bp	basepairs
BW	biological warfare
CE	capillary electrophoresis
CFU	colony-forming units
C. freundii	*Citrobacter freundii*
CPT	cycling probe technology
CZE	capillary zone electrophoresis
ddATP	dideoxyadenosine triphosphate
ddUTP	dideoxyuridine triphosphate
DNA	deoxyribonucleic acid
EA	*Erwinia ananas*
EB	ethidium bromide
EC	*Erwinia carotovora*
EH	*Erwinia herbicola*
E. intermedia	*Escherichia intermedia*
EOF	electroosmotic flow
EU	*Erwinia uredovora*
FT	field trial
LIF	laser-induced fluorescence
Kb	kilobases
nt	nucleotides
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNaseH	ribonuclease H
s.d.	standard deviations
SSC	standard saline citrate
TG-1	*E. coli* TG-1 strain
Tm	melting temperature
TPL	tyrosine phenol-lyase

Introduction

Cycling Probe Technology (CPT) is an isothermal DNA detection scheme (ID Biomedical patents, 1-3) which is well-suited to an automated microchip format. The CPT reaction uses a labelled co-linear RNA/DNA chimeric probe which is complementary to a particular target DNA sequence (1). Upon hybridizing to the target sequence, regions of the probe comprised of RNA form a RNA/DNA duplex structure with target DNA [see Figure 1]. The segment of RNA/DNA duplex is a substrate for a thermostable RNaseH, which degrades the RNA region and releases cleaved probe products. The presence of target DNA sequence is therefore indicated by the conversion of full-length probes into cleaved probe products.

CPT probes can be labelled 5' or 3' with radioisotopes, chromophores or fluorophores, and cleaved CPT products can be separated from intact probe by electrophoresis. In this work CPT probes were made against *Erwinia herbicola*. *Er. herbicola* is a field-trial simulant for aerosolized biological warfare (BW) agents, and is used in the development of rapid BW identification capabilities at DRES. For this work, CPT assays were performed in microtubes and the resulting CPT products were identified using conventional capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) [Beckman, Canada]. An aliquot of the assay was separated by capillary electrophoresis; the smaller fluorescent CPT products (14 to 16 nt) pass the LIF detector at a different time interval than the intact fluorescent CPT probe (28-31 nt). The appearance of fluorescent CPT products positively identifies target DNA in the test sample. The number of targets (chromosomal copies, or organisms) in a sample may then be calculated back from the fluorescent signal.

The proposed microchip biosensor currently under development for DRES requires customized assay formats and biochemical reagents (e.g. CPT, fluorescence-based immunodetection) to optimize the performance of the device. This work is necessary in order to develop and redesign CPT probes that will eventually be used in the microfluidics format. Materials and information from this work will be transferred to Dr. Jed Harrison at the University of Alberta, to perform CPT-CE on the microchip. In this format, both the CPT assay reaction and the separation of CPT products will be performed on the same microchip.

The goal of this contract was to optimize the CPT assay and CE-LIF detection to identify the presence of *Erwinia herbicola* in various samples, in preparation to transfer the reagents to

microchip format. Attempts were made to optimize the CPT assay format as to probe sequence, reagent constituents of the assay, and the minimum time required to obtain reproducible and detectable products. CE-LIF detection was optimized as to buffers, run times and capillary conditions. In both cases the goal was to adapt the CPT assay and CE-LIF detection to the minimum time required to maintain high sensitivity and specificity of identification of the test organism.

Theory of Cycling Probe Technology

Cycling probe technology (CPT) is an isothermal DNA detection method developed by ID Biomedical (patents, 1-3) that can be well adapted to various automated processes and diagnostic kit designs. Its ease of experimental design allows it to be of great potential in the rapid diagnosis of several infectious and genetic diseases. Its rapidity and ease of use also makes it an ideal technology to identify the presence of biological warfare (BW) organisms. Approaches under development are expected to expand the utility of CPT for identification of all genomes.

1. The CPT Reaction

The CPT reaction scheme is outlined in Figure 1. A colinear DNA-RNA-DNA sequence-specific probe of 24-30mers is synthesized: the RNA component consists of 4 ribonucleotide residues and the probe is labelled at the 5' end (Figure 1.1). The assay is based on the differential hybridization of DNA to the target sequence. The probe is allowed to hybridize with sample DNA hypothesized to contain the complementary target sequence (Figure 1.2). The hybridization product contains a DNA-RNA duplex, which is sensitive to cleavage by RNase H (Figure 1.3). The enzyme cleaves the probe at the rN_4 ribo sequence and the cleaved products, having a lower Tm than the intact probe, melt off the target at the reaction temperature (Figure 1.4), leaving the target sequence available for binding of more probe (Figure 1.5). The cleaved products can then be detected by various strategies depending on the 5' or 3'-label, such as autoradiography, fluorescence or colorimetric detection.

The main advantages of CPT as a DNA detection method are a) it is an isothermal reaction, which lends itself to easy automation; b) it is rapid, taking only 30 minutes for the reaction itself; c) there is a linear accumulation of product (unlike amplification strategies which are exponential) which lends itself to accurate quantitation; d) lack of target amplification resolves potential cross-contamination problems which can occur with PCR-based strategies; and d) only one enzyme and one probe are required, unlike conventional PCR strategies which rely on at least two primers and

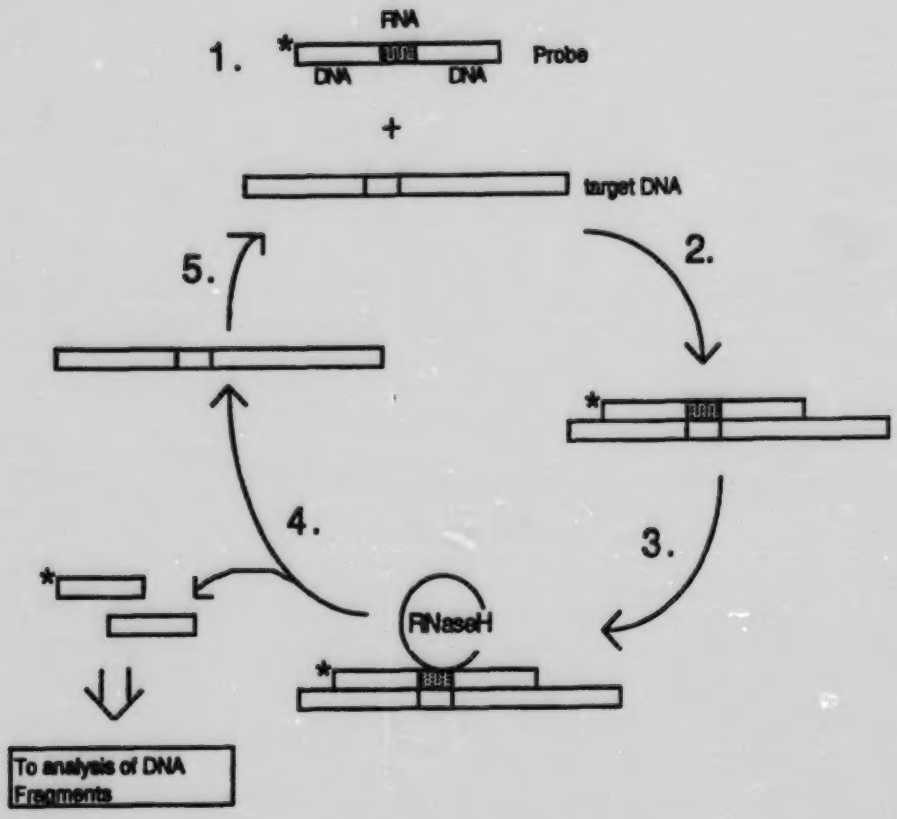


Figure 1. Cycling Probe Technology. A sequence-specific probe (24-30 nucleotides) is synthesized so that an internal stretch of 4-6 ribonucleotides (RNA) is inserted into a single-stranded DNA probe. Step 1. The probe, which is labelled (*) with fluorescent tags, is incubated with target DNA. Step 2. The probe hybridizes with the target sequence and forms a segment of RNA/DNA duplex. Thermostable RNAseH attaches to the RNA/DNA duplex region and cleaves the RNA. Since the hybridization temperature of the resulting cleaved products are lower than the reaction temperature 58°C, the products dissociate from the target sequence (step 4) and the target sequence then become available to hybridize with more CPT probe. Accumulated labelled product is then identified by LIF capillary electrophoresis.

possibly more than one enzyme (as in the case for RT-PCR) (Duck *et al.* 1990; Bekkaoui *et al.* 1999). These properties make CPT ideal for automation and diagnostic kit design for rapid assay formats.

2. Specific Theoretical Considerations of CPT

Although the CPT reaction is theoretically straightforward and elegant in its approach, in practice several considerations belie its simplicity. Perhaps the most demanding aspect of the CPT assay is the design of the probe itself. The probe is limited to relatively stringent design conditions based on the target sequence. The probe must be extensively tested to ensure it has high sensitivity and specificity towards its target. It must be noted that because no target amplification is involved, the sensitivity of CPT tends to be lower than traditional PCR-based strategies. However, as the reaction kinetics are linear as opposed to exponential for PCR, it is far easier to quantitate, as the assay product accumulation remains in a linear dynamic range for a longer time than in exponential-based kinetics. Different detection approaches can also be incorporated, depending on the probe design.

2.1. Probe Design Strategy from Genetic Databases

Probe design strategy is dependent on the number of sequences that have been determined for a particular organism of interest. Probe design begins with a survey of all known sequences of the target gene. Ideally gene targets should be multi-copy to enhance detection sensitivity; this strategywas used to enhance detection of Mycobacterium tuberculosis in sputum samples using the 36-bp direct repeat region, from 10000 to 250 cell-equivalents (Beggs et al. 1996). Alternately, a good target gene would be distinguishable either by its strict presence or absence, or by certain mutations that are present in the target gene but absent from similar genes in other organisms. In this regard, antibiotic resistance genes make good CPT target genes.

Gene sequences are surveyed for the presence of (A)4 islands. RNase H preferentially cleaves (A)4 islands, but sequences containing 1 C or G in the island are acceptable (F. Bekkaoui, personal communication). Poly(A) islands are also less susceptible to cleavage by endogenous ribonucleases compared to poly(C) or poly(U) tracts (Duck *et al.* 1990; Bekkaoui *et al.* 1999), a factor which must be considered when using a probe in a crude sample lysate. Candidate target genes would ideally contain a high frequency of (A)4 islands to increase the probablility of obtaining a useful CPT probe. However, the frequency of (AAAA) occurs only once in every 256 bases, thereby reducing the number of available sites to approximately 0.4% of the target

organism's genome. This limitation may also rule out otherwise useful sequence candidates which may not contain an (A)4 sequence.

After a number of candidate genes are tabled, the next step is to evaluate the sequences on either side of the (A)4 islands in the gene for specific temperature and structural parameters. The AAAA island should be a minimum of 5 bases from the 5' end. Generally a 28 to 30 nucleotide sequence is chosen such that the (A)4 island is centred in the candidate sequence. The melting temperature (Tm) is determined for the entire candidate sequence; the Tm can be based on the Wallace rule (Tm = 2x(AT) + 4x(GC)), the %GC rule, the nearest-neighbor rule, or based on salt and formamide concentrations. In practice we take the Tms derived from either the nearest-neighbor rules, or from the formamide and salt concentrations (0% formamide and 50 mM salt), as these conditions better reflect the CPT reaction conditions. The Tm of the entire sequence must be higher than the CPT reaction temperature, which is usually between 55-65 °C, so that the probe will remain hybridized to its target. Ideally the candidate probe sequence contains a 3' C or G anchor to facilitate zippering during hybridization. However, GC-rich sequences are not recommended because of their extreme base-pairing stability. Approximately 50% GC content is ideal, although previous usable CPT probes have ranged from 28-63% GC (Beggs *et al.* 1996; Bekkaoui *et al.* 1999).

The candidate sequence must also be screened for its ability to form interstrand dimers and intrastrand hairpin structures. (This determination may be performed either before or after the Tm analyses.) The presence of theoretical intrastrand structures is acceptable as long as a) the energy of formation is greater than zero at the reaction temperature (*i.e.*, the formaion of the structure is not favored); b) the Tm of the secondary structure is less than the CPT reaction temperature, such that the structure would be resolved at the increased temperature; and c) the ribonucleotide region is not involved in the structure (*i.e.* base-pairing or loop). Any of these conditions would result in potential endogenous cleavage of the probe by RNaseH (C2 activity, described in Section 2 below).

Once the above conditions are satisfied, the Tms of the sequences 5' and 3' to the (A)4 island of the candidate sequence are calculated. Once cleaved, the products will have a lower Tm than the intact probe. However, to work effectively, the ideal probe should have comparable 5' and 3' Tms (± 2 °C) and the fragment Tms should be significantly lower than the CPT reaction temperature. This ensures that the products will melt off the target at about the same time to regenerate free template. If the 5' and 3' Tms are not comparable, the position along the gene sequence and/or the

length of the probe can be adjusted until they become comparable. In the case of highly asymmetrical sequences, care must be taken to ensure that both strands will melt off the target at the cycling temperature.

After these conditions are met, the probe must be re-checked against the gene sequence database to ensure its uniqueness and eliminate or reduce cross-reactivity with other organisms and genes. In practice, a search comparing the probe sequence to gene sequences with five to seven mismatches should be sufficient to ensure binding specificity and minimal cross-reactivity. Ideally, at least one mismatch should occur in the (A)4 region. Theoretically a 20% mismatch (5-6 bases for a 28-30mer sequence) should be allowed in the probe/target complex before cross-reactivity becomes significant. A 1% sequence mismatch is approximately equivalent to one degree in Tm (Kricka 1992) so this should allow a significant degree of latitude in hybridization reaction temperature to assess target specificity.

In summary, the characteristics of an ideal probe using this design approach are:
a) unique sequence
b) low to no incidence of secondary structures
c) acceptable full-length Tm and comparable 5' and 3' Tms

2.2. Probe Synthesis Considerations

Because the probe is a DNA-RNA-DNA chimera, special attention must be paid to its synthesis. The major difficulty in synthesizing a CPT probe comes from the incorporation of ribonucleotides; unless the 2' hydroxyl is blocked, synthesis can result in a mixture of 2' and 3' ribonucleotide linkages. Typically the 3' DNA component is synthesized first, followed by addition of ribonucleotides and purification after the ribonucleotide addition. Synthesis then continues, adding the 5' DNA component. The probe is then 5' labelled with a chromophore: fluorescein and Cy-5 sulfoindocyanine dye have been used. The probe may be labelled during synthesis by incorporating a labelled nucleotide (fluorescein nucleotide), or post-synthesis by chemical methods (Cy5-N-hydroxysuccinimide ester labelling of a 5' amino-modified end of the probe). The probe can also be enzymatically 3' labelled after cleavage from the synthesis resin, with biotin, ^{32}P or another chromophore, depending on its end use. The easiest method of 3' labelling is by addition of nucleotides by terminal transferase. The major difficulty in labelling post-synthesis (5' or 3') can be expected to be decreased yield of final product, as labelled product must be purified further. However this is not insurmountable as amounts of probe used per assay are typically very small.

2.3. Sensitivity and Specificity

A useful assay and detection method must show both high sensitivity and high specificity towards its target. The sensitivity of the CPT assay depends on several factors, including the method of detection, which is based on the 5'-label (or 3'-label) used; the sequence of the probe; and the overall CPT reaction kinetics. The probe specificity is largely sequence-dependent but can be to some extent modified by manipulating assay conditions.

Sensitivity of the CPT assay is determined by its turnover number, the number of probe molecules cleaved per target molecule (Beggs *et al.* 1996). For a 30 minute reaction, this turnover number is approximately 1000 probe molecules cleaved per target molecule (Beggs *et al.* 1996). The turnover number can be increased either by increasing the concentration of RNaseH added to the reaction in order to detect small amounts of target DNA (Beggs *et al.* 1996) or by increasing the assay incubation time. Alternately, sensitivity can enhanced by larger numbers of target sequence per genome, such as in multiple repeat sequences. While repeat sequences are relatively common in eukaryotic cells, they are relatively uncommon in most bacterial or viral species, with a few exceptions (notably the 36-bp direct repeat sequence from *Mycobacterium tuberculosis*).

Candidate CPT probes can be assayed against a synthetic target, which is generally the complement to the candidate probe, and against purified genomic targets and crude lysates to determine probe sensitivity and specificity. There are 4 types of control reactivities, C1 to C4, which must be determined empirically for each probe lot before it can be used in a CPT protocol. Also, it is good practice to run at least the C2 and C4 controls with a CPT assay, to ensure the probe continues to maintain its properties and to minimize assay-to-assay variations due to reaction conditions.

C1 activity measures the amount of auto-hydrolysis in the absence of enzyme, C1 is thus an indicator of probe stability under the reaction conditions used. It can also be used as a measure of contaminating RNase activity during probe synthesis or post-synthesis handling. However CPT probes are not significant substrates for RNases expected to be found in the environment (RNaseA; Beggs *et al.* 1996). CPT probes appear to be stable at room temperature for at least several hours; experiments where probes were left at room temperature prior to cycling showed no significant loss of cycling ability, provided probes were not exposed to both RNaseH and magnesium (F. Bekkaoui, unpublished data). C1 activity can be overcome by using 2'-O-methyl protector groups on the ribose moieties, but 2'-O-methyl oligomers can show increased C4 activity even with

optimization of EDTA and spermine (see below). These oligomers also have an increased Tm (15 °C per 30mer), which may be unfeasible for some CPT applications.

C2 activity is a measure of the amount of probe cleavage in the presence of RNase H and absence of target. It is an indication of the presence of either interprobe interactions (dimer formation), internal folding (secondary structure within the probe that is not resolved by the higher CPT reaction temperature), or both. An ideal CPT probe should show less than 2% C2 in the absence of target; however, in practice some probes work effectively even with high levels of C2 activity in a complex genomic system, perhaps due to the target stabilizing secondary structures or presence of stablilising additives like spermine (Modrusan *et al.* 1998). True C2 activity is determined by comparisons of probe cleavage in the presence of RNaseH and magnesium ion, to probe cleavage in the presence of RNaseH, magnesium ion and EDTA. The majority of cleaved probe product produced would be expected to be derived from cleavage of the middle bond in the ribo region. However, as there are five potential RNaseH cleavage sites in a CPT probe, it is possible that C2 cleavage may occur preferentially at a different site in the probe than in the presence of target; a sensitive detection system such as capillary electrophoresis may be able to resolve such differences.

C3 activity determines the stability of the probe in the probe-target complex in the absence of RNaseH. In some instances the target itself may act as a ribozyme to cleave bound probe. Although this is rare, it might be expected to happen with potential ribozyme catalytic sequences. Generally C3 activity is not determined unless there is reason to suspect target-mediated probe cleavage in the absence of enzyme.

C4 activity determines the specificity of the probe in the presence of RNaseH and heterologous target DNA or background DNA. The mechanism of C4 activity is thought to be due to non-specific binding of probe and RNaseH to heterologous DNA (Modrusan *et al.* 1998). C4 activity is determined in the presence of heterologous DNA from both related and unrelated species. Depending on the use of CPT, in the absence of complete sequence information for the majority of organisms, it is imperative that the candidate probes be tested in as wide a range of species as possible to determine the probe's C4 activity.

The ability of the probe to detect the target sequence in the target species is also measured, to determine the sensitivity. In practice, the C1, C2 and C4 activities in the CPT assay must be low

to negligible to obtain a true signal, for a probe to qualify as a CPT probe. A low to moderate level of C2 activity may be acceptable if it can otherwise be stabilized. Addition of EDTA (50 μM), spermine (2 mM) or a heterologous synthetic target (3 ng) to stabilize formation of secondary structures within the probe can resolve low to moderate C2 activities and improve the probe's efficiency (Modrusan *et al.* 1998). This may also be the reason for no C2 variability of certain probes in the presence of crude lysates (F. Bekkaoui, personal communication). C4 activity can also be minimized by including 2 mM spermine and 0.5 mM EGTA, or 50 μM EDTA, to the CPT reaction to decrease non-specific probe and enzyme binding.

As hybridization is fundamentally dependent on temperature, the CPT reaction temperature can be manipulated to enhance specificity of the reaction. As long as there is at least 1 mismatch in the ribo region of the probe, theoretically the reaction can be carried out at an elevated temperature, to screen out related sequences. However the useful CPT temperature range is limited to between 55 and 65°C. Below this range, non-specific hybridization increases, while above this range the enzyme may become inactivated, or the probe may be prevented from hybridizing to target.

3. Detection of CPT Products

Because CPT is a hybridization event that does not amplify the signal exponentially, sensitivity of target detection by the assay may be somewhat limited compared to other DNA amplification strategies. As noted above, the turnover number is approximately 1000 probe molecules cleaved per target molecule in a 30 minute reaction (Beggs *et al.* 1996). Increasing the turnover number is required to increase assay sensitivity, either by adding more RNaseH or incubating for longer periods. However increasing incubation times is not feasible when the goal is to provide a rapid assay format (less than 5 minutes). Therefore to a large extent the sensitivity of the CPT assay will depend on the sensitivity of the method used for detection of probe products. The ideal detection method must then need to be able to detect attomoles (10^{-18} mole) to zetamoles (10^{-21} moles), or the picomolar concentration range, in order to detect one target molecule, given the current turnover number for the assay.

The probe products can be detected using a variety of methods depending on the 5' or 3' label. Generally some sort of separation of product from intact probe is required, since the intact probe is also labelled. Products can be separated electrophoretically by traditional polyacrylamide gel electrophoresis or by capillary electrophoresis. Probe products can also be separated by interactions with various ligands that have been previously incorporated into the probe (for

example, magnetic separation using streptavidin-coated beads can separate biotin-labelled intact probe from cleaved probe, or enzyme immunoassay [Bekkaoui *et al.* 1999]). Capillary electrophoresis, the method of choice adapted to microchip format, will be considered below as the primary product detection method.

In traditional molecular biology, labelling with ^{32}P or another radioactive isotope 5' (polynucleotide kinase labelling) or 3' (terminal deoxynucleotidyl transferase labelling) is considered the most sensitive method for detection. ^{32}P-labelled CPT probes have been able to detect 600-6000 molecules of synthetic target, and 4000-10000 molecules of genomic target under ideal conditions for mecA945 probe (F. Bekkaoui, unpublished data). Because most prokaryotes do not contain highly repetitive sequences, molecules of genomic target generally correspond 1:1 with the number of chromosomal copies, or the number of organisms, present in a sample. However, repetitive sequences have been shown to enhance sensitivity: a target against *M. tuberculosis*, containing multiple repeat sequences, further enhanced the sensitivity of the isotopic assay from 10000 to 250 cell equivalents with a signal:noise ratio of 2:1 (Beggs *et al.* 1996). However, most diagnostic assays cannot be expected to be performed under ideal conditions, and clinical diagnosis laboratories are often not equipped to handle radioactivity. Currently the fluorescent and colorimetric detection methods that have been developed for CPT are 10- to 100-fold less sensitive than radioactive labels (F. Bekkaoui, unpublished), but these detection methods are more likely to be encountered in diagnostic laboratories. Despite their lower sensitivities compared to radiolabelling, fluorescent and colorimetric methods are adequate detection methods when sample is not limiting. However, chemiluminescent probes, which approach or surpass the sensitivity of radioactivity, can be effectively utilized (Voyta *et al.* 1995).

Sample preparation is also important for maximal effectiveness of the CPT reaction and hence detection of probe products. For genomic samples, the most efficient probe cleavage occurs with genomic target fragments of 0.5 to 2 kb in length; longer genomic fragments increase the probability of non-specific probe and enzyme binding and increased background signal (Modrusan *et al.* 1998). In crude clinical isolates, endogenous nucleases might be expected to digest genomic DNA to the appropriate length after initial lysis, so no further sample manipulation would be required. However, the extent of endogenous digestion would need to be controlled to prevent total degradation of target in clinical samples. Environmental samples (from air, water or soil) might require further sample processing (*e.g.* sonication to appropriate lengths, or protease or nuclease treatment) before assay to maximize sensitivity.

4. Capillary Electrophoresis as a Detection Method for CPT Assay

Capillary electrophoresis (CE) is rapidly gaining popularity as a fast, inexpensive and sensitive detection method for most types of biological molecules. The separation of analytes is based on the mass/charge ratio of each analyte; the migration times are indicative of the presence of the analyte under defined conditions, and with appropriate method development (peak height or area analysis) the technique can give quantitative as well as qualitative information. The sensitivity of detection of CE approaches the picomolar concentration (zetamole) range when using laser-induced fluorescence to detect fluorescently-labelled analytes. This sensitivity is at least equal to the sensitivity of radioactive detection, without the hazardous and environmental concerns of handling radioisotopes. Capillary electrophoresis also readily lends itself to adaptation to microfluidic formats. The combination of both these factors, as well as its ruggedness, makes CE a promising detection method for CPT assays.

CE detection for CPT assay requires some careful probe and method design considerations. In capillary gel electrophoresis (CGE), separation is based on the size of the analytes due to the sieving effect of the gel and the constant charge/mass ratio of the nucleic acid analytes. However, gels are not robust as a separation matrix, due to degradation with time and batch-to-batch variations during manufacturing. Therefore a more robust CE separation, one that can withstand rugged handling, is required. In free-solution capillary zone electrophoresis (CZE) separation is based on the mass/charge ratio of the analytes. As both intact and cleaved probe carry roughly the same charge/mass ratio, resolution of the species in free solution will be poor unless the mass/charge ratio of one of the analytes is modified, usually by adding a neutral or positively charged species to exert a "drag" on one of the analytes. By adding biotin, digoxigenin or another neutrally-charged molecule to the intact probe on the opposite end to the fluorophore, migration time of the intact probe is slowed relative to that of cleaved probe, thus providing better resolution in free solution.

5. Current and Potential Uses of CPT

CPT appears to have almost ideal utility in clinical diagnosis. The main advantages of CPT compared to other gene detection/identification strategies is speed, assay simplicity and robustness. Therefore this technology can be easily adapted to situations where non-specialist personnel can use it to diagnose disease or detect harmful organisms.

5.1. Diagnosis of Bacterial Infections of Clinical Significance

The rising incidence of infections from antibiotic-resistant strains of common bacteria has highlighted the necessity of rapid and accurate diagnostic testing in the clinical setting (Waldvogel 1999). Although these bacteria are generally harmless in healthy people, they can be lethal to potentially immunocompromised hosts (very young children, the elderly, or hospitalized patients). The difference between antibiotic-resistant *vs.* antibiotic-sensitive bacteria of the same species in many cases amounts to the simple presence or absence of a specific resistance gene. It may also be important to distinguish between borderline-sensitive and resistant bacterial strains, to select appropriate antibiotic therapy (Bekkaoui *et al.* 1999). This hence provides a rapid yes/no answer to better tailor treatment of the infection to the needs of the patient. CPT is thus ideal for detecting such differences between antibiotic resistant and sensitive bacterial strains.

Currently CPT has been adapted to detect the mecA gene in methicillin-resistant *Staphylococcus aureus* (MRSA) (Bekkaoui *et al.* 1997; Cloney *et al.* 1997; Bekkaoui *et al.* 1999); the *vanA* and *vanB* genes in vancomycin-resistant enterococci (VRE) (Modrusan *et al.* 1996); and the 36-bp direct repeat sequence from *M. tuberculosis* in clinical isolates (Beggs *et al.* 1996). In all instances the specificity and sensitivity of detection in laboratory studies approached 100% when compared to conventional susceptibility testing and PCR strategies currently used for diagnosis. ID Biomedical has diagnostic kits under development for detection of these organisms. With the recent appearance of vancomycin-resistant *S. aureus* in North American hospitals (Rotun *et al.* 1999; Smith *et al.* 1999), rapid identification of antibiotic-resistant organisms is now even more crucial to appropriate diagnosis and treatment of infectious disease.

5.2. Detection and Identification of Biological Warfare Organisms

CPT has great potential utility in identifying the presence of biological warfare (BW) organisms during a military or terrorist attack. Rapid and accurate identification of the attacking organism can mobilize an effective means of treatment or prevention of infection before the disease organism can infiltrate the body and exert significant morbidity or mortality (Franz *et al.* 1997). Its ease of use, robustness of reagents and potential for automation and kit design therefore make CPT easily adapted for test equipment to be run by military personnel otherwise inexperienced in molecular biology techniques.

Diseases of potential military and terrorist significance include anthrax (*Bacillus anthracis*), tularemia (*Franciella tularensis*), plague (*Yersinia pestis*), brucellosis (*Brucella* sp.), and the

Western, Eastern and Venezuelan variants of equine encephalitis (members of the alphavirus genus of the Togaviridae family) (Franz *et al.* 1997). The incubation times of these organisms can range from a few days to a few hours depending on the levels of exposure; for most, only a few organisms (10-8000) are required to cause disease (Franz *et al.* 1997). Although experimental vaccines for these organisms exist, they may not always be completely protective (Franz *et al.* 1997). Hence rapid identification will allow medical personnel to administer timely prophylactic or curative measures to military and civilian exposure.

6. Adaptation to Microfluidics Format

Because CPT is isothermal and linear in its reaction kinetics, it readily lends itself to adaptation to a microfluidics format. Miniaturized analytical systems, or "laboratories on a chip" are becoming increasingly important for research and diagnosis (Colyer *et al.* 1997; Knapp *et al.* 1998). Briefly, the reaction is carried out in a reaction chamber, then a sample plug is electrokinetically injected into a separation chamber and analytes are separated by capillary electrophoresis. The analysis time is thus greatly decreased from hours (for conventional detection methods), to a few minutes. The benefits of microchip technology have been discussed elsewhere (Colyer *et al.* 1997).

The adaptation of CPT to a rapid assay for microchip format requires several considerations:

1. Stability of reagents, possibly for several hours or days at ambient temperature;
2. Reduction in reaction volume to accommodate the very small volumes typically used in microfluidics;
3. Reduction in assay time to obtain maximum sensitivity in a minimum amount of time;
4. Contiguous CPT and CE buffer systems for smooth transitions between reaction and separation;
5. Efficient separation of intact and cleaved probe products within a short (less than 5 cm) capillary length;
6. Maximizing both sensitivity and specificity while reducing the length of time of the overall process;
7. Possibly incorporating several probes within one assay to maximize throughput or to confirm detection of the organism.

Reagent stability must be considered during microchip design, as temperature-stable reagents will greatly reduce the complexity of the final microchip design (*e.g.* whether sample or reagent cooling will be required). Reagent stability is a relatively simple area to address. The original CPT assay described used *E.coli* -derived RNaseH (Duck *et al.* 1990). The current assay uses RNaseH

derived from the thermophilic bacterium *Thermus thermophilus* (Bekkaoui *et al.* 1996). In studies performed at ID Biomedical, *T. thermophilus* RNaseH was shown to be stable for extended periods at ambient temperature with no significant loss of enzyme activity (F. Bekkaoui, personal communication). CPT probes were also shown to be active in CPT assays after storage at ambient temperature for 6 to 8 hours, as long as they were stored in the absence of RNaseH (which would potentially increase C2 activity) or magnesium. An important consideration in assessing probe stability is that two parameters, probe activity and fluorophore stability in the reaction buffer, must be examined. For example, fluorescein is notably susceptible to photobleaching and is unstable at a basic pH. Fluorophores must also be tested for stability in the assay reagents.

Reduction in assay reaction volumes is necessary for accommodating the microfluidics format. Current assay conditions at ID Biomedical use 50 to 100 µL assay volumes. However, typical reaction volumes in microfluidics are at the nL scale. Reducing assay volumes for the microchip may have the effect of decreasing detection sensitivity and possibly evaporation and clogging problems at the elevated temperatures used for the assay. However, reducing assay volumes correspondingly decreases the amount of reagents consumed, a potentially critical fact in isolated situations.

The most important challenge to adapting CPT to a microchip format may be to reduce the assay time to a minimum while maintaining both high sensitivity and high specificity of the assay. The sensitivity is currently limited by the reaction turnover number (approximately 1000 for a 30 minute reaction). As accumulation of probe products is linear, a five-minute assay would reduce the turnover number to less than 200. Reducing the assay time to a rapid format (*i.e.* five minutes or less) will thus require either an exquisitely sensitive detection method, assay modifications, or (likely) both. The presence of heterologous DNA in FT samples is likely to interfere with the sensitivity, as C4 activity may be increased or the reaction itself may be inhibited. Probe design may relieve this factor to some extent, but these issues demand testing.

Efficient separation of products on the microchip is likely to be addressed by both prudent probe design as described above, and by modifying separation conditions. As always there will be a compromise between speed of analysis and detection sensitivity. Gel systems in a microchip can be used for separation, but there are often significant batch-to-batch variations and susceptibility to degradation of the matrix, especially at elevated temperatures. Capillary zone electrophoresis in a chip thus is the better method. Incorporating multiple numbers of tags such as biotin in CPT

probes should result in sufficient CZE resolution of intact and cleaved probe species even within short capillary lengths. Different probe modification strategies, using various 3' tags added by end-labelling, will be used to address this area.

A contiguous buffer system for CPT assay and CE detection increases the robustness of the format and reduces complexity of the system and thus the overall chip design. This aspect will be addressed during assay optimizations. Again buffers must be chosen for maximum sensitivity and specificity while retaining speed and robustness. One important point is that modifying CPT reaction conditions can exert a significant effect on CE separations for a contiguous buffer system. Additives such as spermine, used in assays to reduce C4 activity, can have a significant effect on electroosmotic flow (EOF), by reducing or reversing it by modifying the charge on the capillary wall. This can be used to advantage in separations to increase species resolution, but care must be taken that it does not unduly compromise speed of CE analysis. Magnesium, required for RNaseH activity, has also been shown to have significant effects in CE separations by modifying the EOF within the capillary.

The possibility of using several probes in one assay must be considered for independent confirmation of the test organism. To date, combining several probes in one assay at once has not been tested. A multichannel design that can handle several single assays at once may circumvent this problem to some extent, depending on the intended use of the channels (dedication to different probes for the same organism *vs.* dedication to one channel for different organisms). As reliable confirmation depends on multiple tests for the same organism, if the second intention is followed then the assay will require optimization yet again to accommodate more than one probe per reaction. This is not impossible, as other detection strategies, notably PCR, can use several different primer sets at once in the same sample with appropriate reaction modifications (Wong et al. 1994). This possibility is not within the scope of this contract, but the point is made for future considerations in adaptation for microchip formats.

Results

1. Design of Suitable CPT Probes for *Erwinia herbicola*

The known genes of *Er. herbicola*, obtained from Internet-based searches of the Genbank and EMBL databases, were surveyed for sequences that would make suitable CPT probes (Table 1). Of 46 available sequences (as of March 1999), 3 genes (beta-glucosidase [EHBGLA], tyrosine phenol-lyase [ERWTPL], and dimethylallyltransferase [EHETZGENE]) were determined to have sufficiently high frequencies of (A)4 islands to be analysed further. The genes were subsequently analyzed for suitable probe sequences (Table 2). Candidate probe sequences were generated from these 3 genes as outlined in Methods and Materials. The candidate sequences were then compared to the gene sequence databases using the GCG FINDPATTERNS and BLAST programs, for specificity against the target organism (Table 3). During the FINDPATTERNS search, the EH7 and EH9 probe candidates were found likely to highly cross-react with sequences in the tyrosine phenol lyase gene of both *Citrobacter freundii* and *Escherichia intermedia* (4 and 2 mismatches for EH7 and EH9 respectively). EH7 and EH9 were thus not investigated further. The remaining candidates were then synthesized by IDT (Integrated DNA Technologies, Coralville IA) and screened in CPT assays.

2. Quality of CPT Probes

5'-Fluoresceinated or Cy5-labelled CPT probes obtained from IDT were assessed by UV-CE and LIF-CE for purity as a quality control check. Probes obtained from IDT were found to be of acceptable quality. Figure 2 shows typical UV and LIF electropherograms obtained from EH probes (EH8 in this example). The UV probe profiles typically consisted of 1 major peak that ranged between 95 and 100% by area. The LIF probe profile usually showed 2 peaks--a main peak and a trailing shoulder, comprised of incompletely denatured species. The areas of the main and trailing peaks corresponded to the majority of intact probe species. This pattern was seen for both fluorescein and Cy5-labelled probes. Probes stored at -20 °C were remarkably stable with respect to the RNA sequence with storage; electropherograms performed on EH8 probe samples after extended storage and handling showed no significant differences in peak profiles (Figure 3). However, probes stored for extended periods at -20 degrees exhibited decreases in fluorophore signal in CPT assays compared to probes stored at -70 degrees (not shown).

Fluorescein-labelled EH8 probe was further modified in-house by 3'-biotin labelling using TdT and biotin-16-ddUTP. A range of 3' labelling ratios were tested. Using a 2:1 biotin:probe ratio

Table 1. Frequencies of A_4 islands in Gene Sequences from *Erwinia herbicola*

Gene Locus	Name	Size	# A_4 islands	Frequency
EHTNPAGEN	tnpA	857	4	4.6
AF006625	tm protein	5061	51	10.1
S50513	tpl	1631	22	13.5
EHU80202	16S rRNA	1454	2	1.4
EHU93355	ppsA/ydiA/aroH	2701	10	3.7
AF071231	pPATH Avr	1522	17	11.2
AF071524	pPATH hsvG	2350	18	7.7
EHHRC	hrc complex	3750	28	7.5
EHU54764	pgi	501	0	0
ERWPHEAA	pheA	906	11	12.1
ERWIPDC	ipdC	2576	7	2.7
ERWICEE	iceE	4902	16	3.3
ERWIAAM	iaaM	1937	6	3.1
ERWIAAH	iaaH	1421	9	6.3
EHU61275	hsvG	2350	18	7.7
AB008150	grosES/groEL	1942	11	5.7
ERWGAP	gluconic acid gene	1134	3	2.7
ERWTUTA	tutA (tpl)	398	6	15.1
EHU25347	tpl locus	5805	41	7.1
ERWTPL	tpl	2378	25	10.5
EHETZGENE	ipt	1500	32	21.3
ERWCRT1B	crtI/crtB	2415	12	5.0
ERWCRTE	crtE	963	7	7.3
ERWCAROTB	crtH	641	0	0
ERWCRTA	carotene complex	6965	58	8.3
ERWCRTS	carotene complex	12753	60	4.7
EHU51208	bioD	723	1	1.4
EHU51207	bioC	756	0	0
EHBIOTOP1	biotin operon	89	2	22.5

Table 1 (continued)

EHBIOTOP2	bioB	1041	2	1.9
EHU38519	bioA	1290	1	0.8
EHU50183	bioF	1158	0	0
EHBGLA	bglA	1535	20	13.0
EHPHEAGN	pheA/tyrA/aroF	3434	19	5.5
ERWAROQ	aroQ	1132	11	9.7
ERWTYRA	tyrA	1012	10	9.9
ERWPTA	pheA/tyrA/aroF	3434	19	5.5
EHY13249	16S rRNA	1239	1	0.8
AB004757	16S rRNA	1449	2	1.4
ERWRGB	rRNA	795	1	1.3
ERWRGA	rRNA	720	1	1.4
EO3857*	gDNA for tpl *	1571	22	14.0
S69174	16S rRNA	120	0	0
AF035010	tyrR	1880	15	8.0
EHU81376	paaA/B/C	3917	42	10.7

Notes:

Frequency = (# A4 islands)/(sequence length) x 1000

* - this gene sequence is patented--included for information only

Table 2. ***Erwinia herbicola*** **CPT Probes**

FULL NAME	ABBREV	SEQUENCE (RNA IN PARENTHESIS)
Beta-glucosidase:		
EHBGL279-28	EH1	5' tga tca gta cct (aaaa) gat att cag ctg
EHBGL610-30	EH2[1]	5' gtt gaa gtt gca ac (aaaa) atc atg gcc gag
EHBGL1044-31	EH3[2]	5' tat tcg cca tca ga (aaaa) tgc ttc aga agt c
EHBGL1468-31	EH4	5' atc atc cgt ggt g (aaaa) tat cag tga tgt tg
Dimethylallyltransferase:		
EHETZGENE858-30	EH5[3]	5' tat aat tat tgg ct (aaaa) agc aga gtg cta
EHETZGENE1175-26	EH6[4]	5' ttg cag atg caa (aaaa) att gga ttt g
Tyrosine phenol-lyase:		
ERWTPL1140-28	EH7	5' cgc agg cag tg (aaaa) ctt cta cca tct c
ERWTPL1224-28	EH8[5]	5' acg cgg ggc gg (aaaa) cct gct ctc gca g
ERWTPL1682-29	EH9[6,	5' cca tga gcg gta (aaaa) aga ttg tct ggt g
ERWTPL1158-28	EH10[7,8]	5' cta cca tct cga (aaaa) acg gtg aaa gag
ERWTPL1751-26	EH11[9]	5' tct cag cgg c (aaaa) gag ttg gtt gtc

Notes:

[1]hairpin with loop Tm = 33°C (not involving ribo region)

[2]weak hairpin with loop Tm = 5°C (1 A of ribo region involved)

[3]loop Tm = 40°C

[4]loop Tm = 64°C

[5]loop Tm = 52°C

[6]has 6 As in ribo region

[7]has 6 As in ribo region

[8]first 10 bases overlap with last 10 bases of EH7

[9]ribo region is in weak loop (Tm = 11°C)

Table 3. Probe Specificity against *Er. herbicola*

From GCG Wisconsin Package FINDPATTERNS search of each sequence in GenEMBL database release 97/4 (July 1997)

PROBE	# MISMATCHES[1]	% MISMATCH[2]	GENE (if < 5)
EH1	6	21.4	
EH2	7	23.3	
EH3	7	22.6	
EH4	7	22.6	
EH5	N/D[3]	N/D	
EH6	4	15.4	*C. elegans* DNA
EH7	4	14.3	*C. freundii / E. intermedia* tpl[4]
EH8	5	17.9	
EH9	2	6.9	*C. freundii / E. intermedia* tpl
EH10	6	21.4	
EH11	5	19.2	

Notes:

[1] forward direction only

[2] % mismatch is calculated by dividing total # of mismatches by length of sequence

[3] N/D = not determined

[4] tpl = tyrosine phenol lyase

resulted in approximately 50% labelling. A 7:1 biotin:probe ratio resulted in over 90% labelling (Figure 4). Further increasing the ratio to 10:1 biotin:probe resulted in near-complete labelling (95-97%) (not shown). All experiments with biotin-labelled probe were hence performed with probe labelled at a 10:1 biotin:probe ratio.

UV-CE analysis of synthetic targets showed approximately 60%-100% full-length species depending on the synthetic target sequence (Figure 5). Targets were used as is with no subsequent purifications.

3. 3' End-Labelling of CPT Probes with ^{32}P

EH probes were 3' end-labelled with (alpha)^{32}P-ddATP using terminal deoxynucleotidyl transferase (TdT). Labelled probe before NICK column purification showed a significant amount of smaller species (Figure 6). Probes were typically over 90% pure after NICK column purification, as seen by the majority of the probe migrating in the upper band on the autoradiogram (Figure 6). Probes were subsequently used as is with no further modifications. Probes typically labelled with specific activities between 1.0×10^3 and 7.0×10^3 cpm/fmol, depending on the sequence of the probe.

4. Screening of CPT Probes by 3' ^{32}P Labelling

4.1. Screening Against Synthetic Targets

CPT assays were performed using 3' end-labelled probes against synthetic targets (complementary sequences to the CPT probes) in TES buffer to determine the C1 and C2 activities. Samples were run in microtubes and analyzed by conventional polyacrylamide gel electrophoresis and autoradiography. Of the probes screened, C1 activity was invariably low (less than 2% in most cases; Figure 7; Table 4) suggesting that the probes were insensitive to autolytic cleavage. However, C2 activity varied with the probes. Most probes showed a C2 activity of less than 10%. However, the C2 activity of EH6 and EH10 (greater than 25%) was significant enough to interfere with quantitation of probe cleavage (Table 4a). Analysis of the predicted secondary structure of EH6 in the OLIGO program showed that EH6 formed a stable hairpin immediately 5' of the ribo region with a Tm of 64 °C (Figure 8a). The predicted secondary structure of EH10 also showed a stable hairpin that involved the ribo region in the loop (Figure 8b). EH8 consistently showed the lowest C2 activity, usually less than 5% in radioactive assays. OLIGO analysis of predicted secondary structure showed that EH8 also formed a stable hairpin structure, that was 3' of the ribo region but did not involve it (Figure 8c).

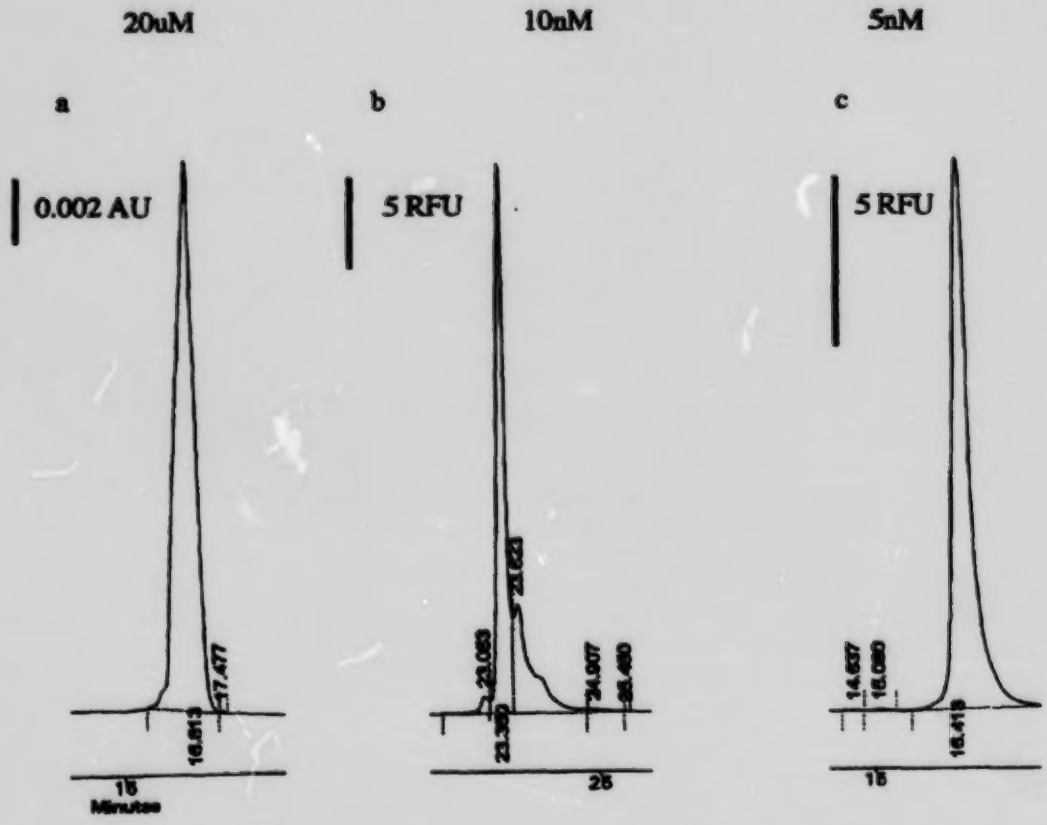


Figure 2. CE-LIF of fluorescein and Cy5-labelled EH8 probes for quality control analysis. Samples were dissolved as indicated in distilled water. Column conditions as described in text. UV detection 254 nm, LIF detection (fluorescein) 488/520 nm, (Cy5) 635/650 nm.

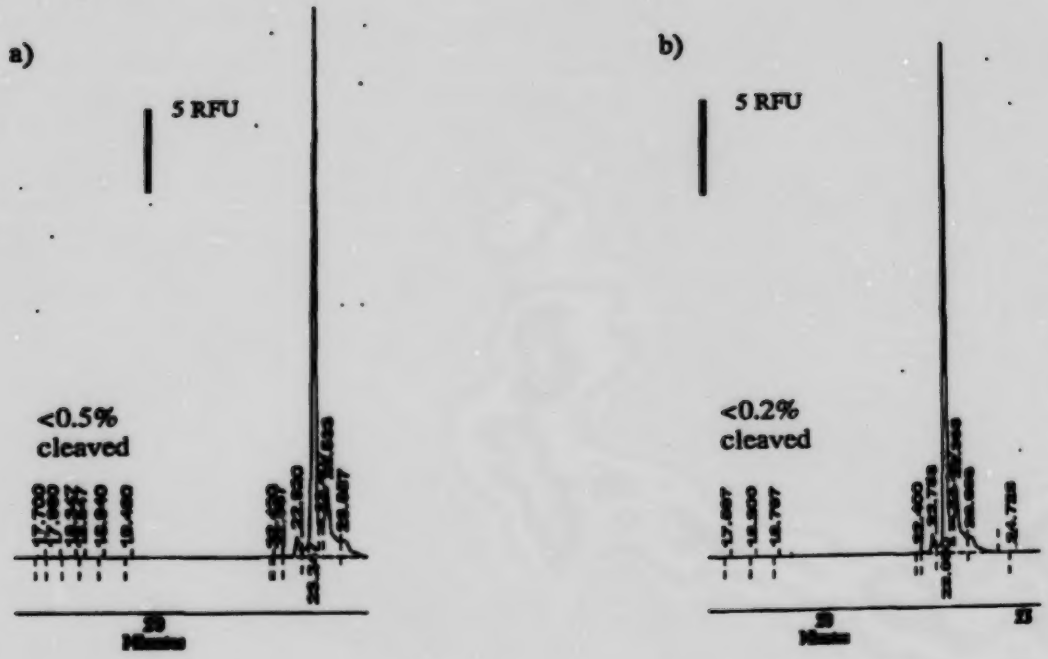


Figure 3. Comparisons of EH8 probe after extensive handling. a) shows an electropherogram of 50 nM EH8 probe that was extensively handled in various experiments. b) shows an electropherogram of 50 nM EH8 probe that was prepared fresh from stock at -70°C. Capillary electrophoresis conditions as described in the text.

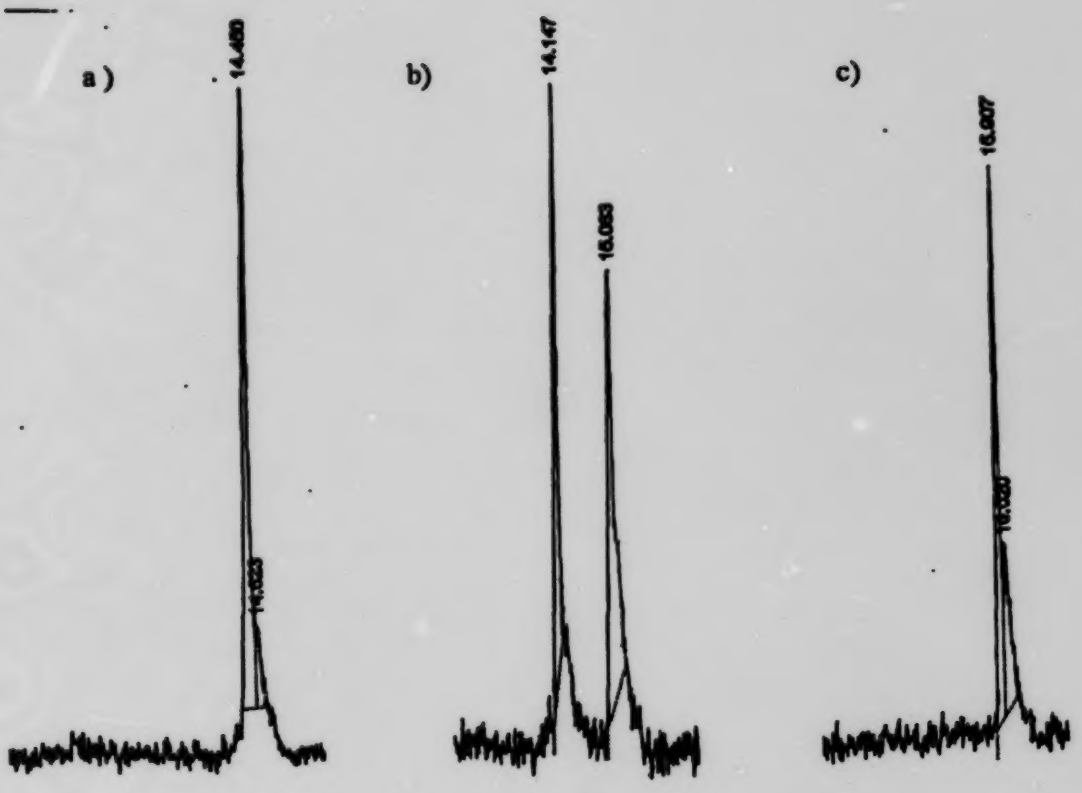


Figure 4. Labelling of EH8 probe with biotin-16-ddUTP. Twenty pmol of EH8 probe was labelled with either 2:1 or 7:1 of biotin-16-ddUTP and terminal transferase as described in the text. Values on electropherograms indicated retention times. Scale, 0.1-0.2 RFU. a) 50 nM EH8; b) 2:1 biotin; c), 7:1 biotin. Capillary conditions: 27 cm x 100 um i.d. Beckman eCAPssDNA100 column, l.d. = 20 cm (reverse polarilty), temp. = 30°C, in Beckman TRIS/borate/7M urea buffer, 7.5 kV x 5 seconds injection (20 ul sample), 300 V/cm separation.

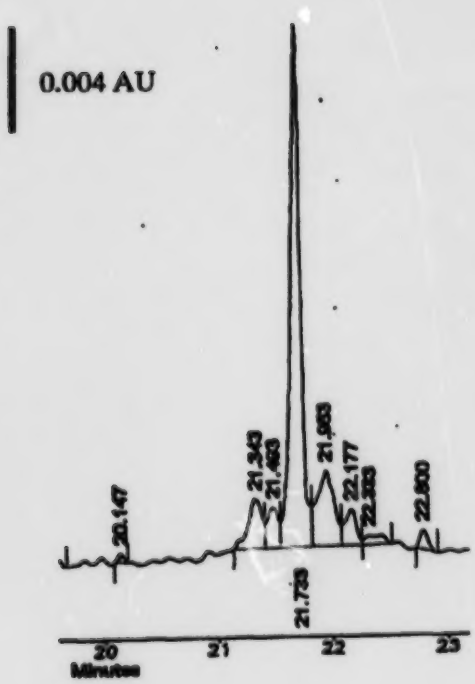


Figure 5. UV-CE analysis of 10 uM cEH8 synthetic target DNA. Synthetic target was made at DRES on a Beckman Oligo 1000 DNA synthesizer and dissolved in water. Conditions: 37 cm x 100 um i.d. Beckman eCAPssDNA100 column, l.d. = 30 cm (reverse polarity). Column temperature 30°C. 7.5 kV x 5 second injection, 300 V/cm separation in Beckman TRIS/borate/7M urea buffer.

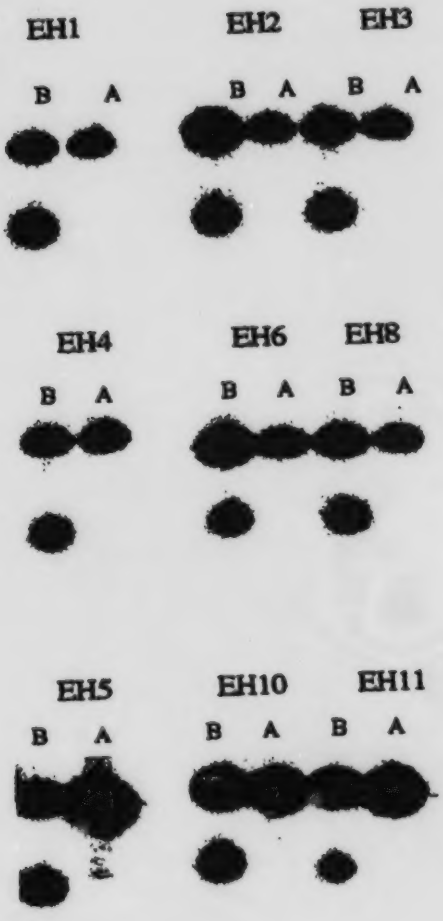
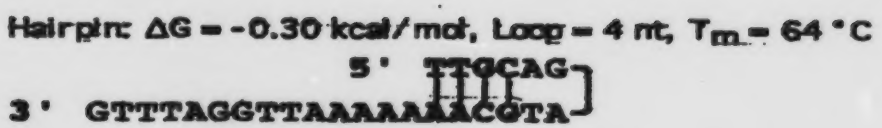


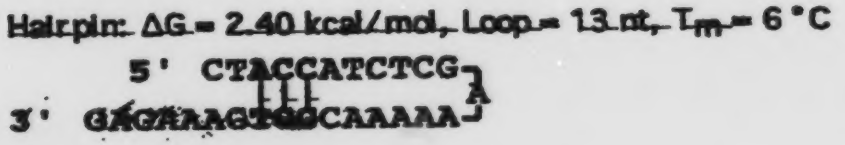
Figure 6. ^{32}P 3' end-labelling of CPT probes for isotopic assays. Probes (2 pmol) were labelled with alpha-^{32}PddATP and purified as described in the text. B = before purification; A = after purification. Specific activities from 500-6000 cpm/fmol.

Figure 7. CPT probe screening with synthetic target. Probes were 3' labelled with ^{32}P as described in the text. Synthetic target samples were run as described in the text. Samples ranged from 3 x 10^6 (1) to 3 x 10^2 (5) molecules. Gels were exposed overnight to obtain autoradiograms. a) EH1; b) EH2; c) EH3; d).EH4; e)EH6; f) EH8; g) EH10; h) EH11. C1 = C1 control; C2 = C2 control; C2+E = C2 control + 10 mM EDTA.

a) EH6 (C2 = 29%):



b) EH10 (C2 = 70%):



c) EH8 (C2 = 6%):

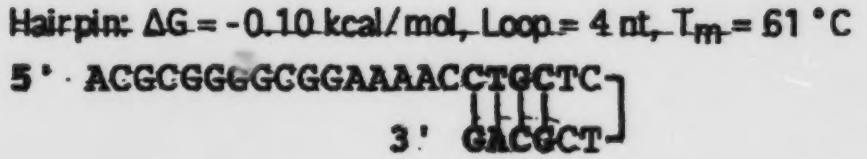


Figure 8. Intrastrand secondary structure analysis of selected EH CPT probes. Probes were analysed in the OLIGO Ver. 5.1 primer design program for Macintosh computers. Tms are calculated for a reaction temperature of 60°C. C2 activities noted in parentheses.

4.2. Screening Against Genomic Targets

Screening of probes against genomic targets began by using partially-purified full-length chromosomal DNA in a basic reaction buffer (20 mM TES pH 6.8, 4 mM MgCl2, 0.05% Triton X-100, 0.01 μg/μL RNaseH). Screening of EH2, EH3 and EH8 probes with full-length DNA showed no probe cleavage at high target concentrations (10^7 copies) (Figure 9). At low concentrations of unsonicated DNA (10^4 copies), EH8 appeared to recognize both *Er. herbicola* and *Er. ananas* DNA specifically (Figure 12).

Sonication of genomic DNA to fragment sizes of 0.5-2 kb relieved the inhibition of cleavage at high DNA concentrations, as did adding spermine (2 mM) and EDTA (50 μM) to genomic target samples, and increasing RNaseH to 0.05 μg/μL per assay sample (Figure 10). Length of target sonication did not influence assay signal (not shown). Screening against sonicated genomic targets showed that EH8 and EH4 showed the most reactivity to specific genomic target. EH8 cleavage was strong in the presence of target DNA, while EH4 cleavage was weaker (Figure 10). EH5 and EH6 (derived from dimethylallyltransferase on the pPATH plasmid) did not appear to recognize target DNA; EH10 showed a very high C2 activity that was not relieved by spermine or EDTA. The remaining probes showed minimal reactivity against either EH DNA or TG-1 DNA (not shown). The probes detected from 3×10^4 to 3×10^5 synthetic target molecules, and 1×10^4 to 1×10^7 chromosomal copies (Table 5). Subsequent studies used EH8 probe as the test probe to develop the assay in both isotopic and CE detection formats for target detection.

One set of experiments examined probe cleavage in two different sodium phosphate buffers, 20 mM pH 6.8 and 10 mM pH 7.0 (Figure 11; Table 6). The target-mediated probe cleavage in phosphate buffers appeared consistently higher in phosphate buffer than in TES buffer; qualitatively, 10 mM phosphate pH 7.0 appeared to give better probe cleavage. As previous CE experiments showed that very little probe was injected onto the column electrokinetically from TES buffer, assays switched to phosphate buffer format.

Another set of experiments used genomic target DNA that had not been denatured prior to assay (Figure 12). The same amount of cutting occurred with DNA that had not undergone a denaturation step prior to assay, suggesting that either the sample DNA was already significantly denatured, or that the probe bound in a triplex structure, thereby displacing the complementary strand. Therefore, sample DNA did not need to be denatured prior to assay in this system.

Table 4. Representative C1 and C2 Activities and % Probe Cleavage for *Er. herbicola* CPT Probes Screened by Isotopic Assay *vs.* Synthetic Target

{a) C1 and C2 activity:

Probe	C1 (% cut)	C2 (% cut)	C2 + EDTA (% cut)
EH1	1.2	6.3	N/A
EH2	0.9	8.1	N/A
EH3	0	7.6	N/A
EH4	0	6.2	N/A
EH6	0	28.6	N/A
EH8	1.5	5.7	N/A
EH10	0	69.9	6.4
EH11	7.4	22.5	5.9

b) % cleavage (uncorrected):

Probe	3×10^6	3×10^5	3×10^4	3×10^3	3×10^2
EH1	93.4	38.6	32.8	18.6	17.2
EH2	93.5	66.9	45.5	47.2	75.7
EH3	96.8	78.5	19.2	13.3	5.6
EH4	88.7	74.1	44.1	77.8	70.4
EH6	84.3	60.9	50	29.5	46.2
EH8	43.0	11.8	6.4	5.0	9.4
EH10	51.7	70.1	60.7	64.9	79.5
EH11	50.3	32.9	23.7	20.2	21.0

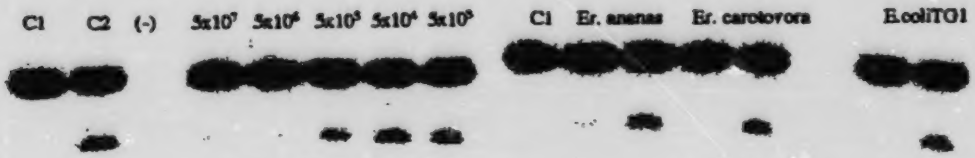
Table 5. Detection Limits for All EH Probes by Isotopic Assay

Probe	Estimated Detection Limit (Synthetic)	Estimated Detection Limit (Genomic)
	(molecules target)	(chromosomal copies)
beta-glucosidase		
EH1	3×10^4	1×10^7
EH2	3×10^4	1×10^7
EH3	3×10^4	1×10^7
EH4	3×10^4	1×10^5
dimethylallyltransferase		
EH5	3×10^5	1×10^7
EH6	3×10^4	1×10^7
tyrosine phenol-lyase		
EH8	3×10^4	1×10^4
EH10	N/D (C2 too high)	N/D (C2 too high)
EH11	N/D	1×10^7

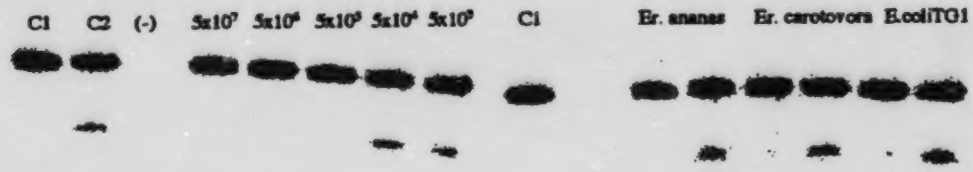
Table 6. Probe Cutting in Phosphate Buffers for EH8 Probe and Genomic DNA (from Figure 15).

Buffer		10 mM phosphate pH 7.0	20 mM phosphate pH 6.8
Sample		Corrected % Cut	Corrected % Cut
C1		0	5.9
C2		0	5.8
EH	5×10^7	28.6	17.0
	5×10^6	20.7	16.7
	5×10^5	16.6	12.2
	5×10^4	33.2	24.1
	5×10^3	18.2	14.9
EA	5×10^6	32.7	22.2
	5×10^3	19.1	15.4
EC	5×10^6	10.2	15.6
	5×10^3	17.0	9.2
TG	5×10^6	14.5	11.4
	5×10^3	8.8	8.9

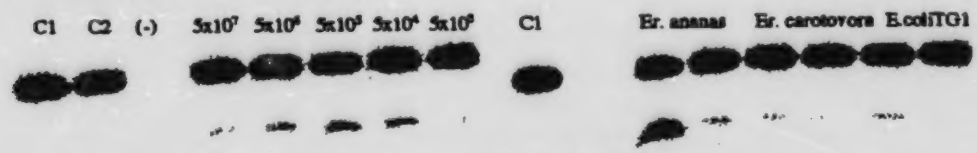
a) 1 fmol EH2



b) 1 fmol EH3



c) 1 fmol EH8



Er. herbicola target

heterologous target

	Sample	EH2 corrected % cut	EH3 corrected % cut	EH8 corrected % cut
	C1	0	0	4.6
	C2	13.8	24.4	5.1
	5.00E+07	2.2	7.9	9.7
	5.00E+06	4.2	5.5	12.9
	5.00E+05	12.4	11.1	19.4
	5.00E+04	13.7	20.9	15.2
	5.00E+03	13.9	22.0	9.1
Er. ananas	5.00E+06	3.0	0.9	29.9
	5.00E+03	13.0	19	9.3
Er. carotovora	5.00E+06	2.0	11.6	5.3
	5.00E+03	15.4	21.2	3.5
E. coliTG-1	5.00E+06	4.9	9.4	10.5
	5.00E+03	16.7	25.0	1.5

Figure 9. CPT Assays with unsonicated genomic DNA. ^{32}P-3'labelled EH2, EH3 and EH8 probe (1 fmol/sample) were tested against genomic DNA as indicated in the figures. Assay conditions: 20 mM TES pH 6.8, 4 mM $MgCl_2$, 0.05% Triton X-100, 0.01 µg/µL RNaseH.

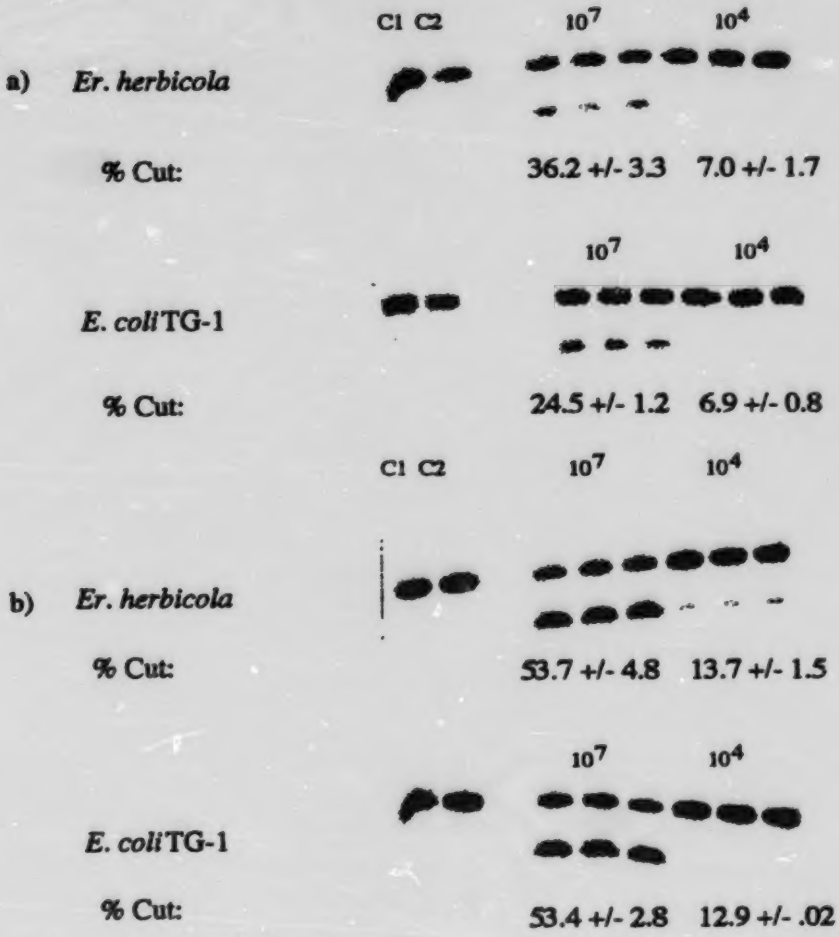


Figure 10. CPT assays with sonicated genomic DNA. Assays were performed using 2 fmol probe/reaction as described in the text. Samples use 10^7 and 10^4 copies of chromosomal DNA as indicated. Values beneat autoradiograms indicate corrected % cuts. a), EH4; b), EH8.

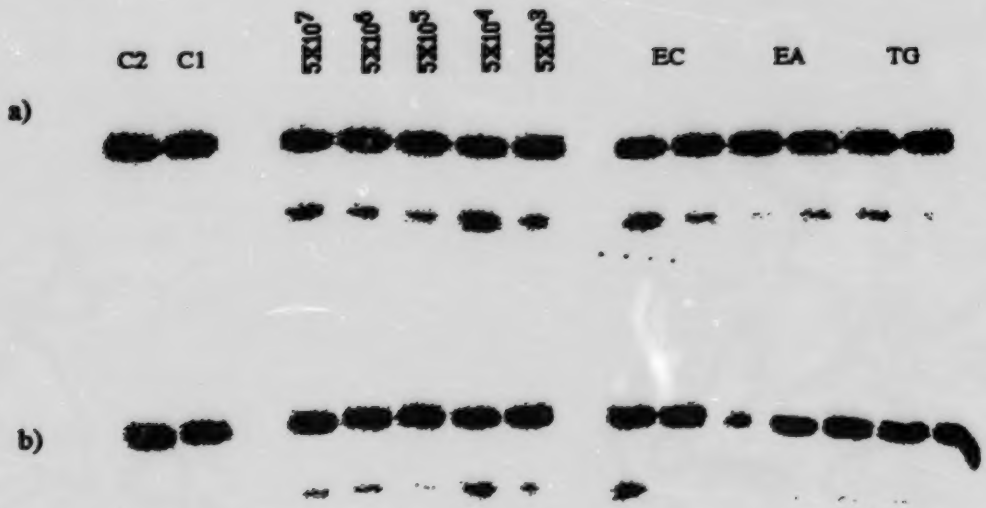


Figure 11. CPT assays in phosphate buffers. CPT assays were performed with 2 fmol EH8 probe per assay and sonicated genomic DNA in a) 10 mM sodium phosphate buffer pH 7.0 or b), 20 mM sodium phosphate buffer pH 6.8.

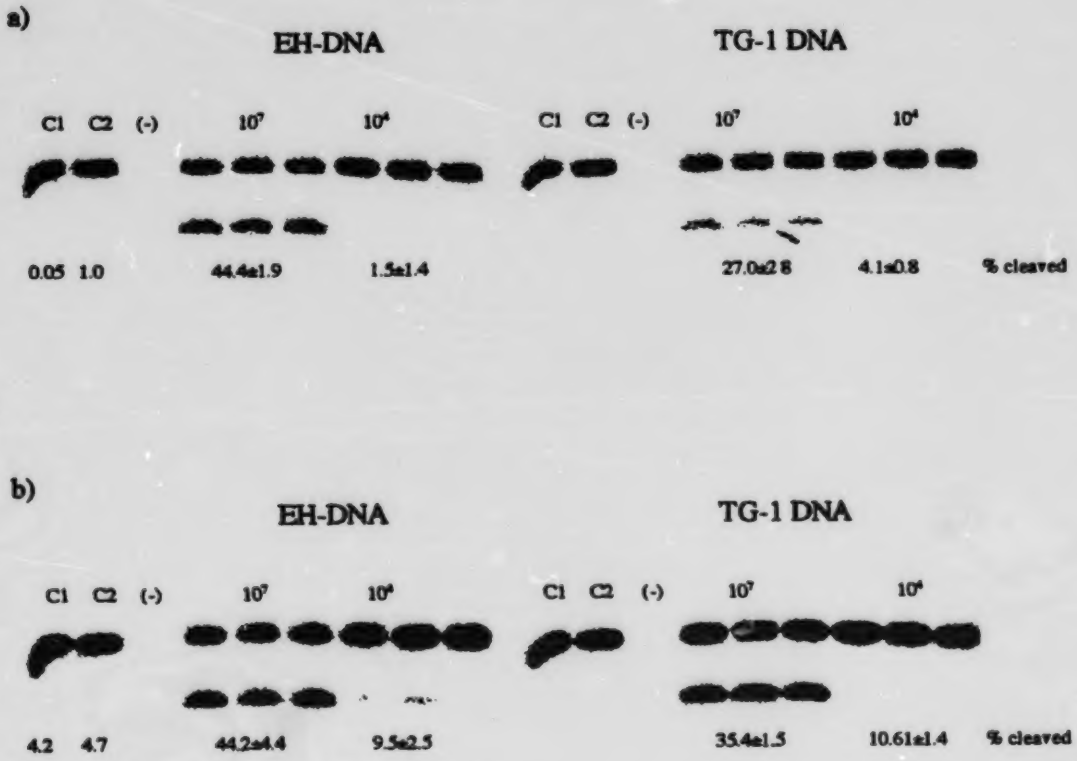


Figure 12. CPT Assays with non-denatured genomic target. a), Genomic DNA samples not denatured prior to assay. b), Genomic DNA samples denatured at 94 °C for 5 minutes prior to assay. Assays performed in phosphate buffer, 2 fmol EH8 per sample. For probe cutting: sample n = 3, average ± SD.

One difficulty encountered during probe screening was the apparent lack of specificity of EH4 and EH8 towards *Er. herbicola* DNA using sonicated target DNA. Various approaches were tried to increase specificity of EH8 to target DNA. We first tried modifying reaction conditions by employing a "hot start/hot stop" technique, where target samples were pre-heated prior to adding cocktail, and stopping dye was added to samples at 60 °C respectively. This "hot start/hot stop" did not significantly improve the specificity of EH8; the probe recognized TG-1 DNA to the same extent as specific target DNA (Figure 13).

EDTA concentrations were adjusted from 0 to 150 μM in an attempt to try to increase probe specificity. Spermine concentration was maintained constant at 2 mM. No significant effect on specificity was observed, as seen by the probe cutting at each concentration (Figure 14).

The effect of spermine concentrations on probe specificity was also addressed. Spermine was varied from 0 to 5 mM while keeping EDTA constant at 50 μM. Spermine inhibited cutting at high probe concentrations (> 3 mM) (Figure 15). Minimal effect on specificity was observed; cutting was the same for both EH and TG-1 DNA.

The assay temperature was changed to attempt to increase specificity of probe for *Er. herbicola* DNA. The theoretical melting temperature of the full-length probe, calculated by the OLIGO program, was 91 °C. As RNaseH maintains activity up to 75 °C, the reaction temperature was varied between 60 and 75 °C. Concentration of target was increased to 10^8 copies to enhance sensitivity. Modifying the reaction temperature did not significantly change the lack of specificity of the probe for EH-DNA. Increasing the temperature resulted in significantly decreased activity of enzyme: compared to assays performed at 60 °C, probe cleavage was decreased by 20% at 65 °C, and by 80% at 70 and 75 °C (Figure 16).

5. CPT Assays with CE-LIF Detection

CPT assays with synthetic, EH and TG1 genomic targets were transferred to capillary electrophoresis detection format using commercially available polyacrylamide gel columns and LIF detection. The transfer required slight modifications from the isotopic format described above. The base buffer was switched to 10 mM phosphate pH 7.0; preliminary fluorescent CPT assays with an ID Biomedical probe (mecA945) in TES buffer showed poor electrokinetic injection of analyte onto the column (Figure 17). To stop the reaction, 50 mM EDTA was used in place of the urea loading dye. Genomic samples were not heat-denatured prior to loading on the column; heat

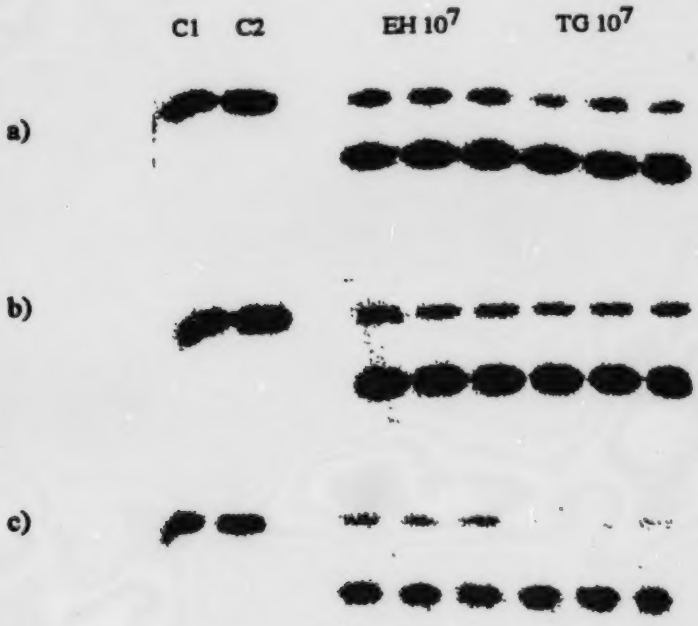
denaturation was found to degrade the signal of the Cy5 fluorophore, most noted in samples with low amounts of target DNA. Also, heat denaturation prior to sample loading would not be possible in the current microchip design.

5.1. Detection Limits of Fluorescent vs. ^{32}P CPT Assays

Detection limits for CPT assays with CE-LIF detection were compared to detection limits of isotopic assays using purified DNA (genomic and synthetic target) and EH8 probe (Table 7). The lower detection limit for genomic *Er. herbicola* DNA was consistenly 10^5 copies with fluorescent assays, using either Cy5 or fluorescein-labelled probes, compared to 10^4 copies for isotopic assays. Interestingly, the lower detection limit of synthetic target was 3×10^4 molecules for isotopic assays, and 3×10^6 molecules for fluorescent assays under current assay conditions. There appeared to be no significant differences in detection limits between the Cy5 labelled and fluorescein labelled probes with regards to target cleavage (Table 7). Representative genomic assays with Cy5 and fluorescein-labelled EH8 probes are shown in Figures 18 and 19. The detection limits were dependent on the sensitivity of detection of the fluorophore. A probe concentration of 10-20 nM per sample (20-40 nM per reaction) yielded adequate fluorescent signal to obtain good signals (S/N >2) for cleaved probe in low-copy target and C2 samples (not shown).

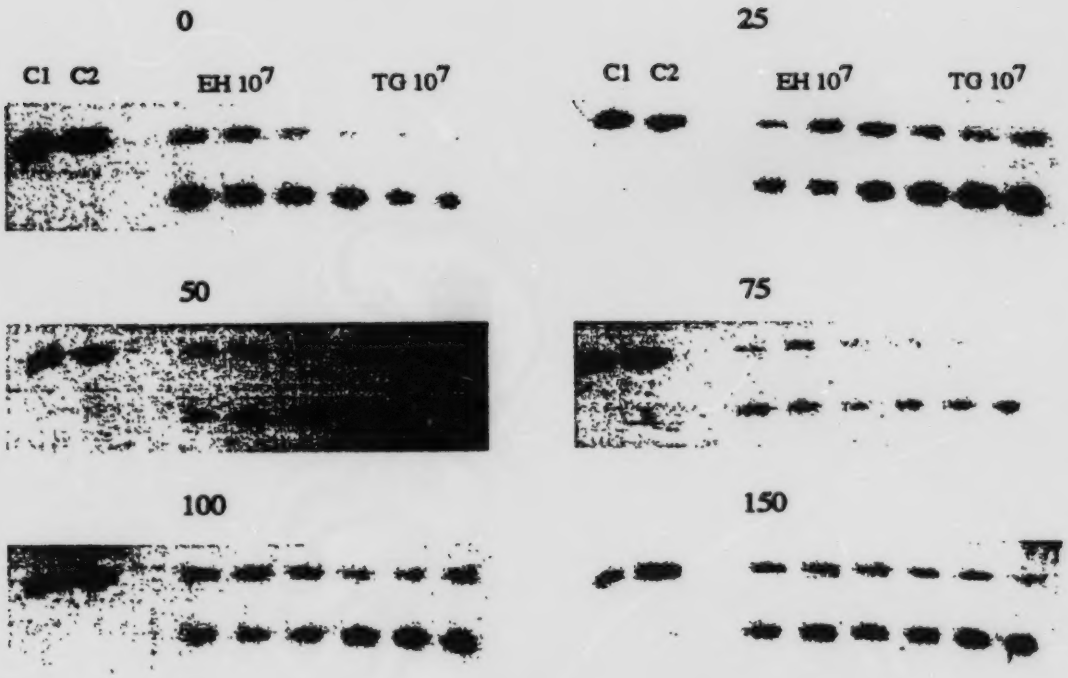
An important consideration was whether C2 cleavage products would be significantly different than target-mediated cleavage products. A comparison of the peak height profiles of C2 cleavage with target-mediated cleavage at 3×10^8 synthetic target molecules for Cy5-EH8 is shown in Figure 20. The peak heights in C2 cleavage gave similar height distributions as seen for target-mediated cleavage, suggesting that RNaseH cleaves in a non-target-mediated manner. Hence the probe products of C2-mediated cleavage was not significantly different from target-mediated cleavage.

One set of experiments using CE-LIF detection, attempted to determine the detection limits in the presence of heterologous non-target DNA (commercially available lambda/HindIII DNA), to determine the extent of interference of heterologous DNA in this system. The presence of heterologous DNA at 5 ng/μL in genomic target assays appeared to reduce the sensitivity of the assay even further, by significantly interfering with target cutting at high target concentrations and increasing C2 activity compared to samples without heterologous DNA (Table 8).



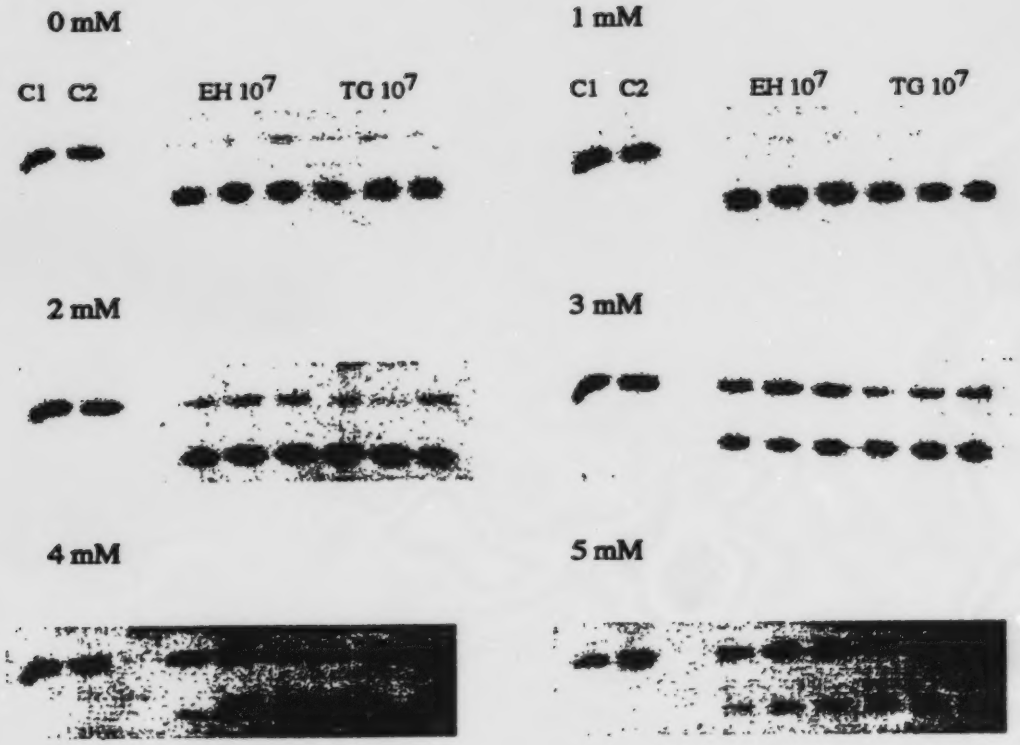
	EH-DNA % cleavage	TG-DNA % cleavage
normal	74.6	82.4
hot start	68.1	77.9
normal	87.7	89.4
hot start	81.5	86.3
hot start	73.4	81.7
hot start/stop	81.3	87.3
hot start	94.2	100
hot start/stop	90.1	98.9

Figure 13. Effect of hot start/hot stop technique in CPT assays with genomic target. EH8 probe (2 fmol/reaction) was tested against EH an TG1 DNA (10^7 copies in triplicate). "Hot start" involved sample pr heating prior to adding probe. "Hot stop" involved stopping the assay at th reaction temperature. a), no heating prior to start or stop; b) hot start onl c), hot start/hot stop. C2-corrected probe cuts for each average are indicat in the table.



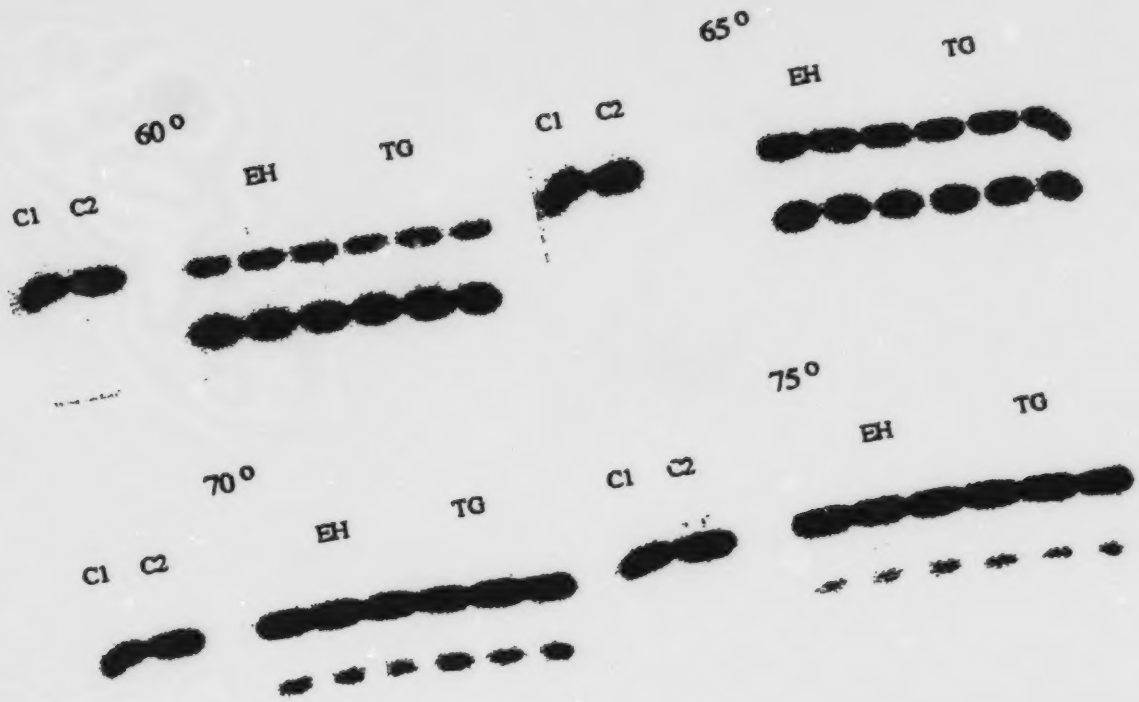
EDTA (microM)	% EH Cleavage	% TG Cleavage
0	72.3	81.4
25	57.2	66.8
50	48.2	64.3
75	49	80.5
100	35.5	87.1
150	60	70.3

Figure 14. Effect of EDTA concentration in CPT assays with genomic target. EH8 probe (2 fmol/reaction) was tested against EH and TG1 DNA (10^7 copies in triplicate) in buffe containing various concentrations of EDTA as indicated. C2-corrected probe cuts for ea average are indicated in the table.



Spermine (mM)	% EH Cleavage	% TG Cleavage
0	90.1	87.2
1	92.9	100
2	70.5	69.6
3	50.7	69.1
4	48.5	55.6
5	41.3	40.1

Figure 15. Effect of spermine concentration in CPT assays with genomic target. EH8 probe (2 fmol/reaction) was tested against EH and TG1 DNA (10^7 copies in triplicate) in buffer containing various concentrations of spermine as indicated. C2-corrected probe cuts for each average are indicated in the table.



Temperature (degrees C)	% Cleavage (EH)	% Cleavage (TG)
60	65.1	86.5
65	44.6	56.4
70	15.3	18.9
75	11.7	12.2
	(avg.)	(avg.)

Figure 16. Temperature effect on RNaseH activity in CPT assays. Assays were performed as described in the text, varying temperatures as shown. Samples used 10^8 copies of sonicated genomic DNA and 20 fmol ^{32}P-labelled EH8 probe per reaction.

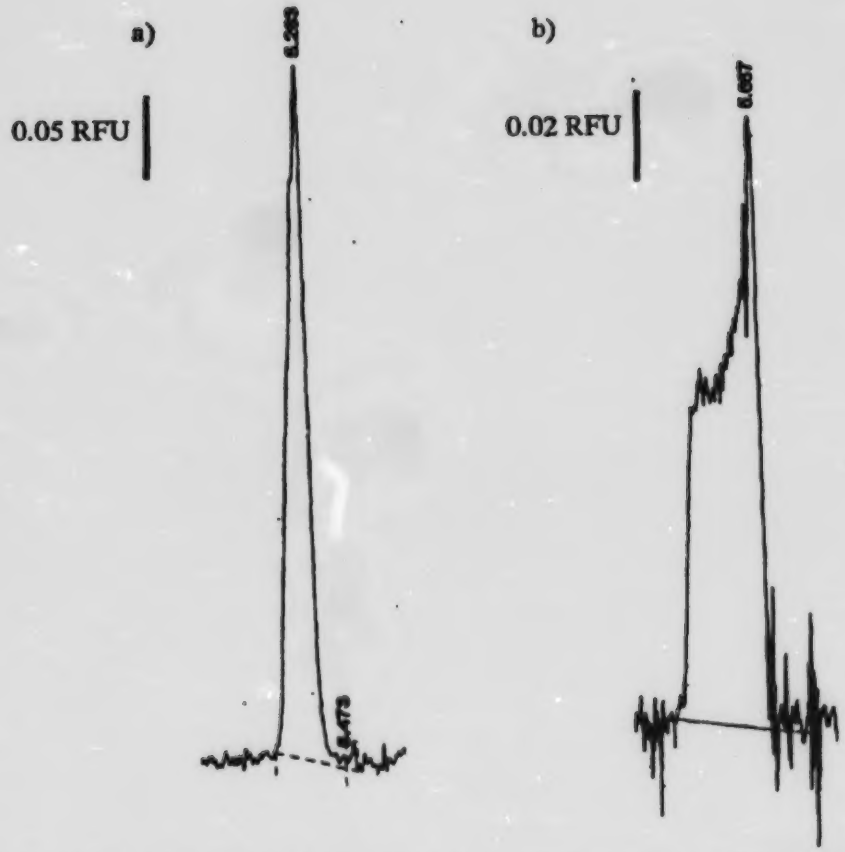


Figure 17. Injection of mecA301-1 onto column: water vs. TES buffer. mecA301-1 in 20 mM pH 6.8 TES buffer (ID Biomedical) was dissolved to 100 nM in a) distilled water or b) 20 mM TES pH 6.8, and sample was electrokinetically injected onto a 37 cm x 100 um i.d Beckman eCAPssDNA100 column. Conditions: column temperature = 30°C, l.d. = 7 cm, normal polarity mode, injection 7.5 kV x 5 seconds, separation 300 V/cm in Beckman TRIS/borate/7M urea buffer.

Table 7. Comparison of Detection Limits for EH8 Probe: Isotopic vs. CE Detection

	^{32}P Assay	CE-LIF Assay	
		Cy5 Label	Fluorescein Label
Synthetic[1]:	3×10^4	$3x\ 10^5$--$3x10^6$	$3x\ 10^5$--$3x10^6$
Genomic[2]:	10^4	10^4-10^5	10^4-10^5

1. Absolute number of molecules target
2. Chromosomal copies

Table 8. Sensitivity of genomic CPT assay in presence of lambda/HindIII DNA. Assays were performed with 5 ng/μL lambda DNA as a heterologous source, using 5' fluoresceinated (non-biotin-labelled) EH8 probe (20 nM/reaction) and *Er. herbicola* sonicated genomic target.

Sample	avg. ± s.d (no lambda)	avg. ± s.d (5 ng/μL lambda)
C2	3.3	32.5
10^7	61.6 ± 9.7	20.7 ± 6.5
10^4	0.7 ± 0.6	4.0 ± 4.8

Table 9. Comparisons Using Internal Standards with Synthetic and Genomic CPT Assays.

a) Cy5EH8 probe with *Er. herbicola* genomic target, coinjected with 50 nM free Cy5 dye. Data based on peak areas. Correction factor based on average Cy5 peak areas.

Sample	Peak Area Cy5 Dye	Correction factor	Raw Cut	Cy5 Corrected Cut
C2	24	1.2	9.6	7.9
10^8	21.5	1.4	19.6	14.5
10^7	22.9	1.3	42.6	33.6
10^6	25.5	1.1	66.9	58.8
10^5	20.3	1.4	9.8	6.9
10^4	37.9	0.8	12.1	15.8
10^3	35.7	0.8	13.5	11
C2	44.4	0.7	7	4.6
10^8	24.7	1.2	18.7	22
10^7	29.1	1.0	51	50.8
10^6	27.6	1.1	74.9	78.7
10^5	8.7	3.3	2.8	9.3
10^4	39.1	0.7	8.2	6.1
10^3	49.6	0.6	8.1	4.7

9b) Cy5EH8 probe with synthetic target, coinjected with 50 nM free Cy5 dye. Data based on peak areas. Correction factor based on average of Cy5 peak areas.

Set 1	Area Cy5%	cxn factor	Raw % Cut (Cy5 corrected)	C2 Corrected Cut
C1	43.5	1.05	0	
C2	36.1	1.27	3.1	
3×10^8	43.4	1.05	100.3	97.2
3×10^6	46.8	0.98	6.5	3.4
3×10^4	49.0	0.93	4.0	0.9
3×10^8	44.3	1.03	97.8	94.7
3×10^6	49.3	0.93	5.3	2.3
3×10^4	52.8	0.87	1.9	0
Set 2				
C1	34.4	1.19	0	
C2	48.1	0.85	9.0	
3×10^8	35.9	1.14	104.1	95.1
3×10^6	40.0	1.02	8.9	0
3×10^4	44.1	0.93	11.2	2.2
3×10^8	41.0	1.00	90.2	81.2
3×10^6	41.5	0.99	13.2	4.2
3×10^4	42.4	0.96	9.4	0.3

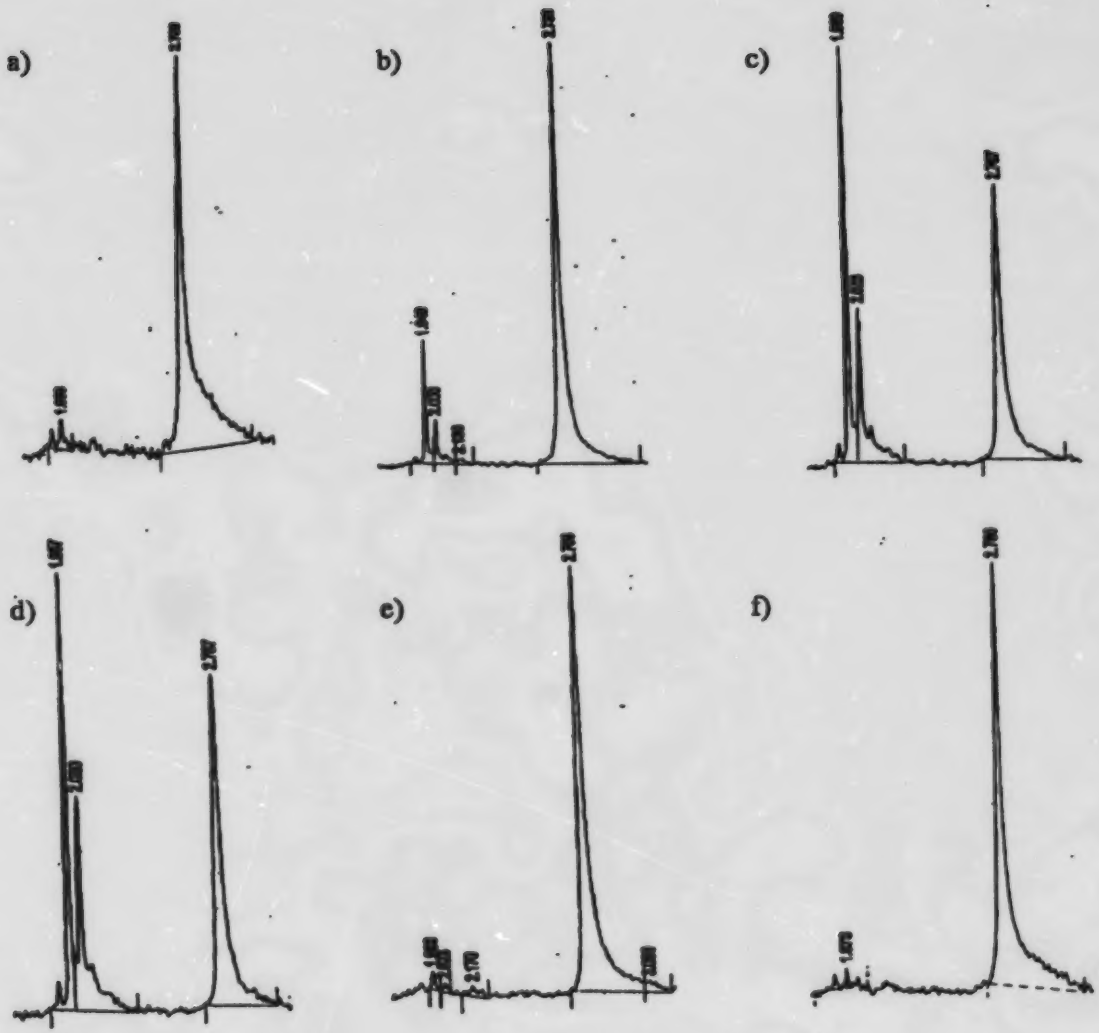


Figure 18. Representative CPT assay electropherograms with Cy5EH8 probe (10 nM) and sonicated *Erwinia herbicola* genomic DNA. Assay and capillary electrophoresis conditions are as described in the text. Values on electropherograms represent retention times in minutes. Values in parentheses represent % probe cleavage (uncorrected) for each sample. The slowest migrating peak is the intact probe. a) C2 control (4.0%); b) 10^8 copies (14.7%); c) 10^7 copies (47.8%); d) 10^6 copies (51.4%); e) 10^5 copies (2.0%); f) 10^4 copies (5.7%)

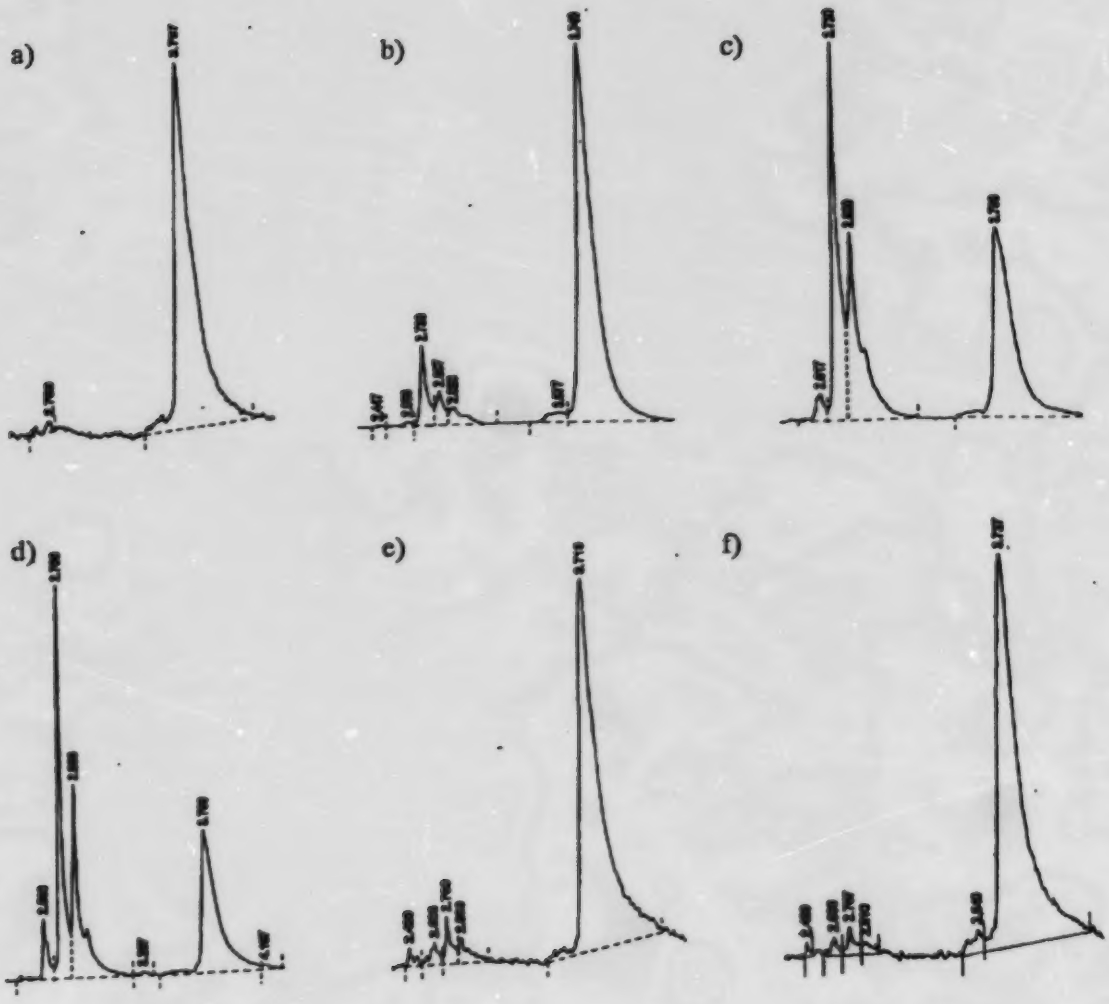


Figure 19. Representative CPT assay electropherograms with EH8-B probe (10 nM) and sonicated *Erwinia herbicola* genomic DNA. Assay and capillary electrophoresis conditions are as described in the text. Values on electropherograms represent retention times in minutes. Values in parentheses represent % probe cleavage (uncorrected) for each sample. The slowest migrating peak is the intact probe. a) C2 control (1.4%); b) 10^8 copies (14.6%); c) 10^7 copies (56.7%); d) 10^6 copies (60.3%); e) 10^5 copies (9.7%); f) 10^4 copies (5.4%)

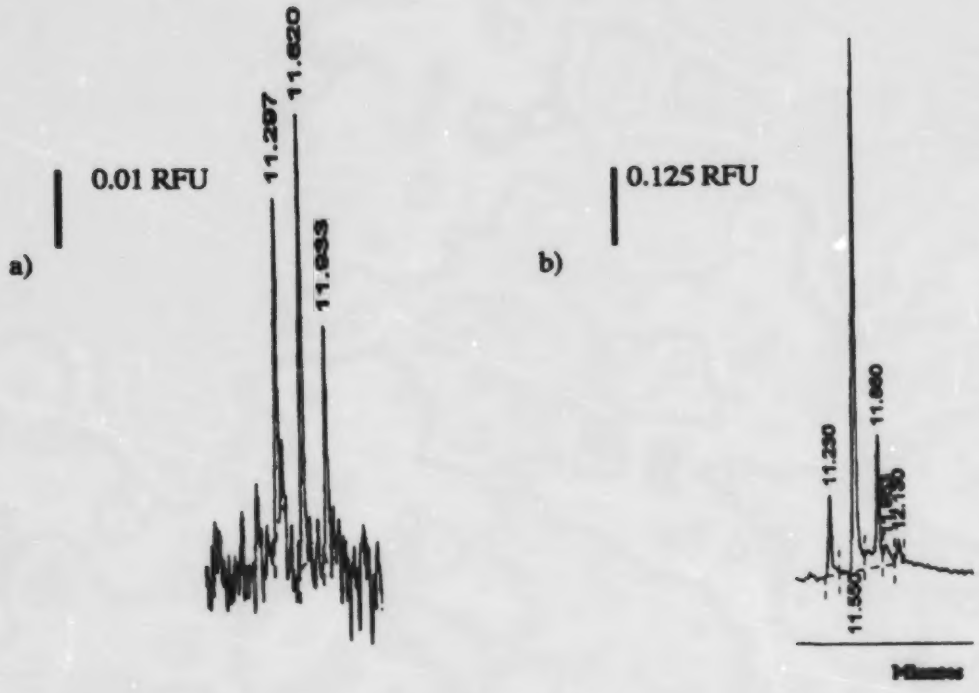


Figure 20. C2-mediated cleavage vs. synthetic target-mediated cleavage of Cy5EH8 probe. 40 nM probe was run with a) distilled water or b) 10^{10} M synthetic target in a CPI assay, and loaded onto a Beckman 27 cm x 100 um i.d. eCAPssDNA100 column. L.d. = 2 cm, temp. = 30 °C, injection 7.5 kV x 5 seconds, separation 300 V/cm in Beckm TRIS/borate/7 M urea buffer.

5.2. Reproducibility of Fluorescent CPT Assays

A series of assays was performed with several different preparations of sonicated *Er. herbicola* DNA to determine assay reproducibility. Each assay was performed in duplicate using 10^8 to 10^3 copies of DNA. Enzyme cutting was inhibited at 10^8 copies compared to 10^7 copies. There was great variability in assay-to-assay cutting for samples at the same DNA concentration. For Cy5EH8 probe, the variability was most pronounced at 10^7 and 10^6 copies of DNA; cutting ranged from 10 to 60% cleavage based on peak areas at 10^7 copies (Figure 21a) based on C2-corrected probe cleavage. Excluding obvious outliers tightened the variability of the assay to less than 20% sample standard deviation at each DNA concentration (Figure 21b). Less variability was seen for the EH8-B probe (Figure 22), although it was still significant. At least some of the variability was attributed to electrokinetic injection used to load the sample onto the column. Studies using internal standards (free Cy5; fluorescein not shown) showed that the internal standard peak areas varied greatly (approximately 10-50%) with electrokinetic injection from sample to sample for genomic target, especially at the extreme high and low concentrations of genomic DNA (Table 9), although both dyes have a similar charge/mass ratio as the probe analytes. The variability was much less for synthetic target samples (Table 9b). However, as the free dye migrated very closely to the first cleaved product peak on the gel column, it was possible that in some samples the area of the internal standard was overestimated. The great variability of sample loading for either fluorescent probe with genomic target suggested that absolute quantitation of DNA by this assay system would be very difficult under the current assay conditions. However, a qualitative yes/no answer or a semi-quantitative answer (rough approximation of CFUs) would be readily achieved. This remains to be confirmed with field-trial samples.

5.3. Studies of CPT Reagent Formulations for Field Trial Use

A series of experiments using Cy5-EH8 probe was designed to test the stability of the probe with prolonged storage at ambient temperature. Cy5-EH8 was examined as the model microfluidic system designed by the University of Alberta incorporates Cy5 detection. Complete synthetic assay cocktail (without spermine or RNaseH) was prepared for Cy5-EH8 and left at ambient temperature (20-22 °C) for up to 6 days. At 0, 1, 2 and 6 days, aliquots were removed for use in CPT assays with synthetic target.

The Cy5 fluorophore proved to be stable in CPT assay cocktail over 6 days; CE-LIF showed no degradation of fluorescent signal of probe at each timepoint (not shown). However the sensitivity of the assay was greatly decreased; the probe lost significant CPT activity in synthetic target assays

Table 10. Time course of Cy5EH8 probe stability at ambient temperature. Probe was stored in complete assay buffer without enzyme. Temperature (20-22 °C) was measured before sample was taken for assay. Assay was performed with duplicate samples.

% Cut against synthetic target (average of duplicates)

Time (days)	C1	C2	3×10^8	3×10^6	3×10^4
0	0	9.1	31.2	1.4	1.5
1	0	3.3	11.0	10.7	9.7
2	0	10.0	7.3	0.7	5.4
6	0	14.6	2.4	0.2	0

Table 11. Time course of Cy5EH8 probe stability at ambient temperature-2. Probe was stored in complete assay buffer with 4 mM spermine, and without magnesium or enzyme. Temperature (20-22 °C) was measured before sample was taken for assay. Assay was performed with duplicate samples.

% Cut against synthetic target (average of duplicates)

Time (days)	C1	C2	3×10^8	3×10^6	3×10^4
0	0	4.5	81.3	0.1	0.1
1	0.9	5.1	64.9	0.7	0.7
2*	3.9	0	98.4	17.0	27.4
4**	0	48.3	51.7	18.2	0

* presence of white precipitate noted--attempted to resuspend prior to assay

** presence of white precipitate noted--no attempt to resuspend

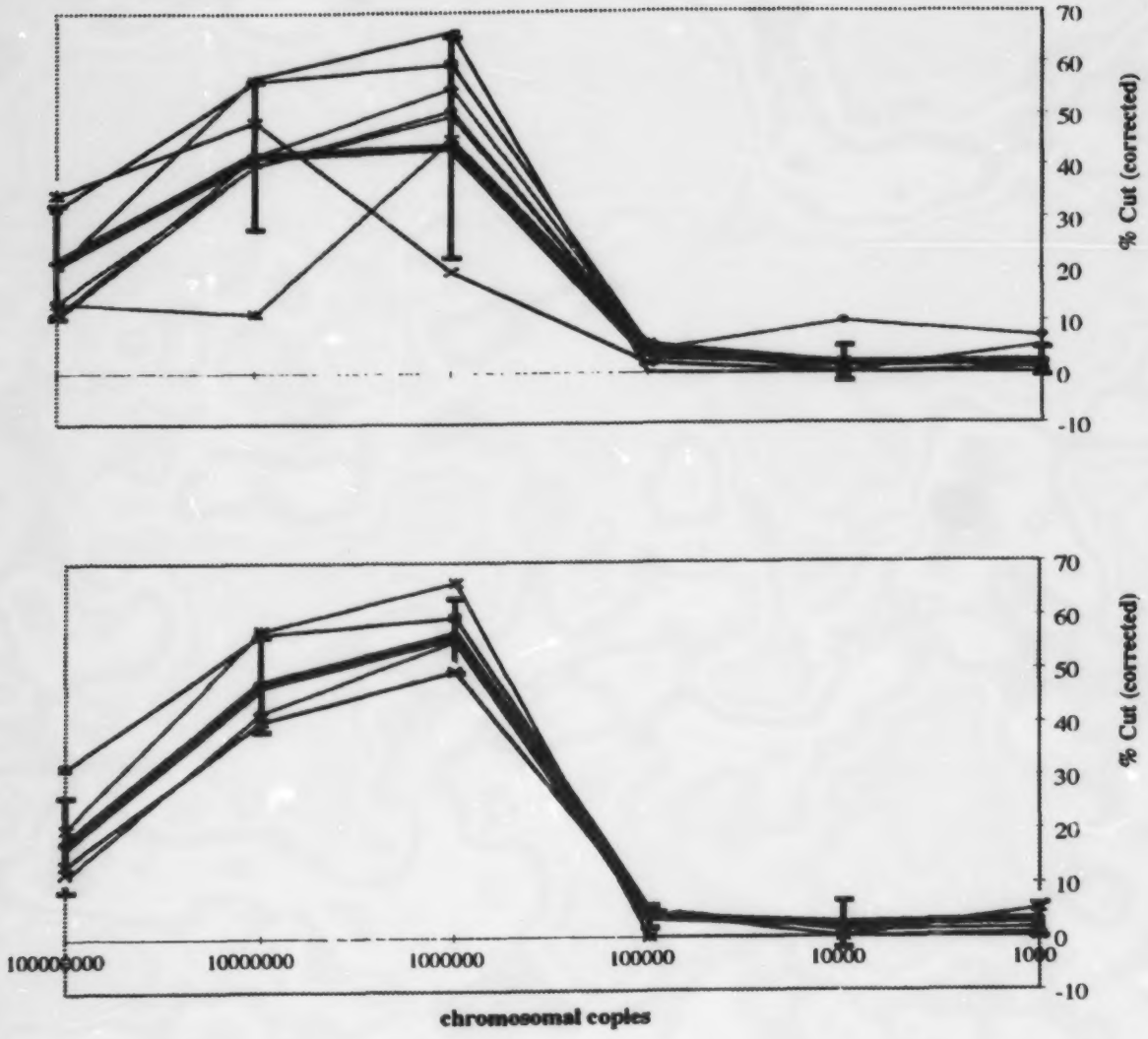


Figure 21. Reproducibility of genomic CPT assays with CE-LIF detection using Cy5EH8 probe and *Er. herbicola* genomic target. Genomic assays were performed as described in the text. Each assay was performed in duplicate. Thick black bar represents average values of CPT assays; error bars, +/- s.d. a), results from 9 separate assays; b), removal of obvious outliers.

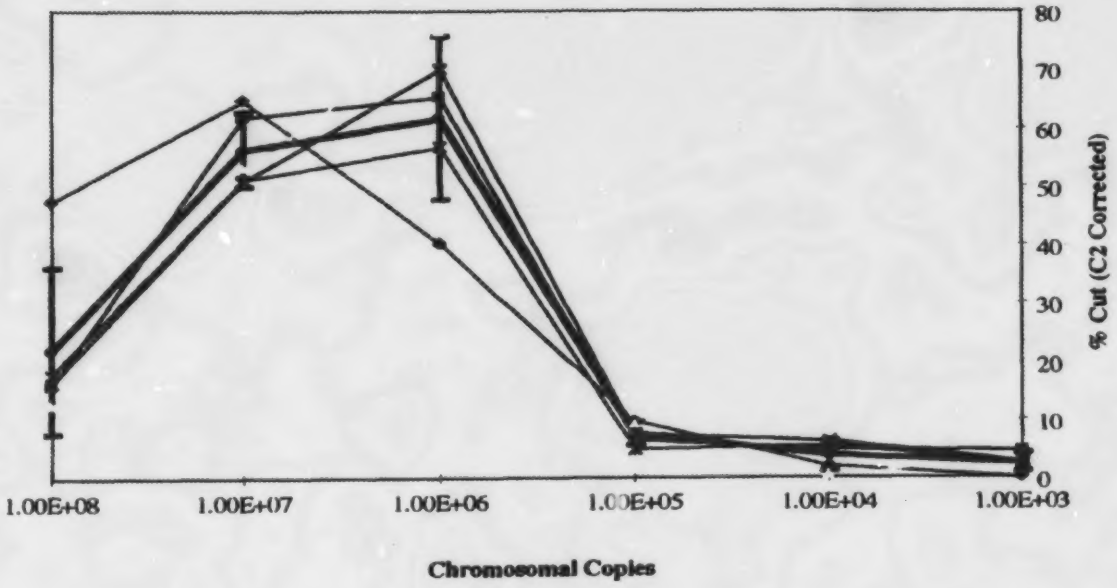


Figure 22. Reproducibility of genomic CPT assays with CE-LIF detection using EH8-B probe and *Er. herbicola* genomic target. Genomic assays were performed as described in the text. Each assay was performed in duplicate. Thick black bar represents average values of CPT assays; error bars, +/- s.d.

after only one day of storage at room temperature (Table 10). C2 activity slowly increased with time of storage at room temperature, from 3% to 15%, thereby interfering with the lower limit of detection of the assay (Table 10). Because the probe was stored in the presence of magnesium, the ion likely stabilized formation of secondary structures. The experiment was then repeated by storing probe in CPT assay cocktail without magnesium; however spermine was added to the cocktail. Magnesium and RNaseH were added just prior to assays. Probe stored in cocktail containing spermine buffer showed decreasing fluorescent signal with time in synthetic target assays (not shown); probe cutting was inconsistent over 4 days (Table 11). After 2 days storage at ambient temperature, a white precipitate had formed, and increased in quantity with further storage. The precipitate was thought to be a spermine-phosphate-probe complex. From these experiments it was clear that the Cy5EH8 probe could not be stored for extensive periods at ambient temperature in a fully reconstituted cocktail.

To try to alleviate the problem of storing probe in wet cocktail, Cy5EH8 probe was prepared in cocktail, dessicated in a Speed-Vac roto-evaporator and stored for up to 6 days at ambient temperatue. Dried cocktail was reconstituted in a 8 mM $MgCl_2$ solution prior to assay. One assay set contained RNaseH in the dried cocktail, while the other assay set had enzyme added at the time of assay. Results from assays where RNaseH was not included in cocktail but was added prior to assay, showed no significant change in probe cleavage while stored in dried cocktail, even after 6 days at ambient temperature; C2 activity was noted to gradually increase, but was still under 10% after 6 days (Table 12). Results from assays where RNaseH was dried in the cocktail, showed decreased cutting and loss of sensitivity compared to control (Table 12). This was thought to be due to the drying conditions used for the enzyme. Previous work by ID Biomedical had shown that cocktail containing RNaseH can be stored lyophilized at room temperature without significant loss in activity. These results suggested that the probe may be readily supplied in a dessicated cocktail, stored at ambient temperature for at least 6 days, and reconstituted in magnesium solution without significant degradation of assay signal.

6. Specificity of Fluorescent CPT Assays

To confirm results noted in isotopic assays regarding specificity of EH8 probe to genomic target, a series of assays was performed using heterologous genomic targets (EH, EA, EC, EU and TG-1DNA). Both Cy5 and fluorescein/biotin labelled probes were assessed, at 2 different target concentrations (10^7 and 10^5 copies). Both probes behaved in a similar fashion to the 5 different targets (Figure 23). At 10^7 copies, the probes appeared to recognize TG-1 DNA preferentially; the

Table 12. Cy5EH8 probe stability after drying and reconstitution. Probe was dried in assay sample buffer without magnesium and stored at ambient temperature (20-22 °C). Cocktail was reconstituted with 8 mM $MgCl_2$ prior to assay. Values are average of 3 samples (s.d.), corrected for C2. (-) RNaseH, probe dried without enzyme, enzyme added prior to assay; (+) RNaseH, probe dried with enzyme.

Sample	no drying	0 hours after drying	24 hours after drying	48 hours after drying
(-) RNaseH				
C2	4.8	2.3	4.8	8.9
3×10^8	48.8 (5.0)	54.0 (2.2)	71.9 (8.0)	81.8 (1.5)
3×10^6	0.4 (0.8)	5.3 (2.7)	4.4 (3.4)	7.0 (2.0)
(+) RNaseH				
C2	6.1	2.0	3.5	7.6
3×10^8	51.0 (1.9)	9.8 (1.1)	25.2 (1.5)	14.4 (1.1)
3×10^6	0.8 (0.6)	3.4 (0.9)	1.9 (0.5)	2.4 (2.2)

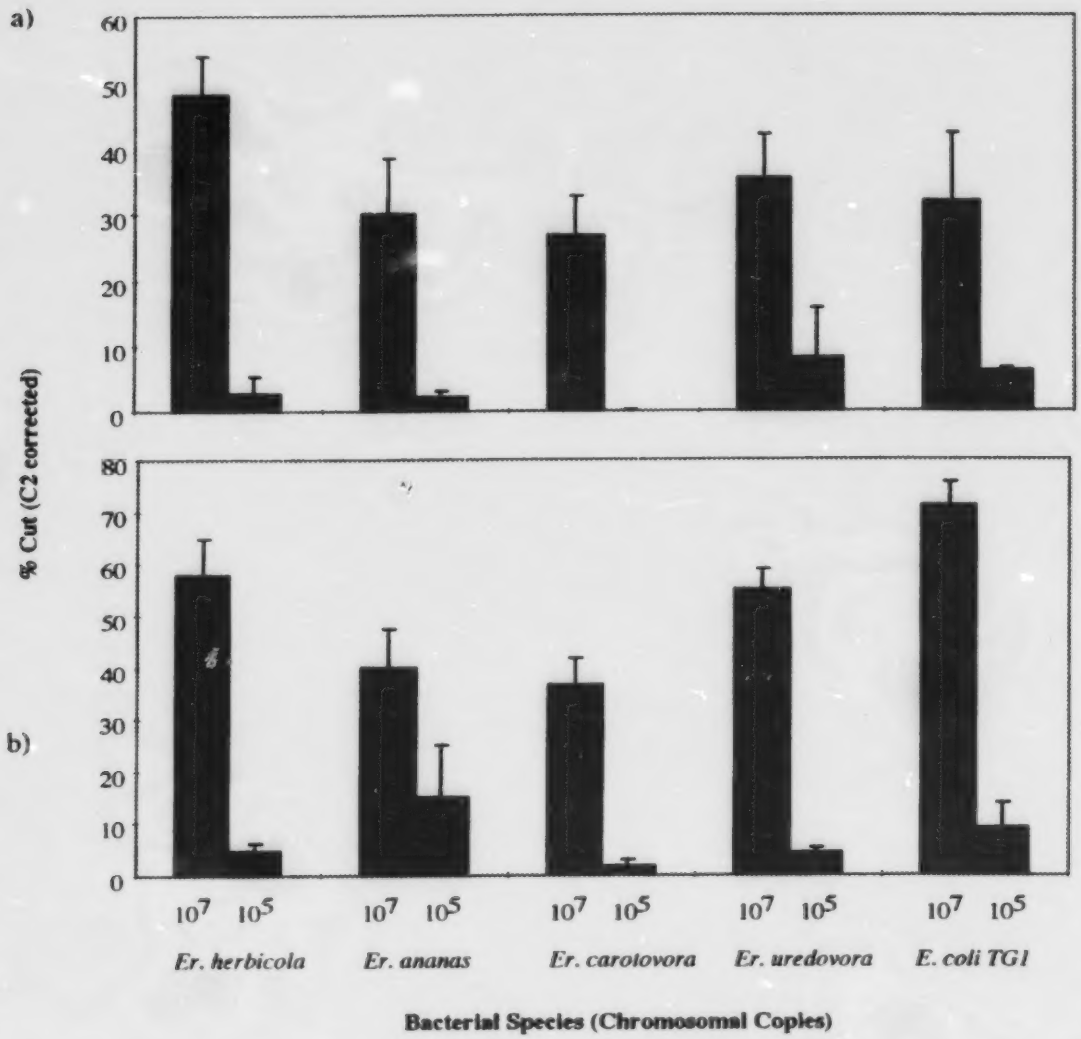


Figure 23. CPT assays with different *Erwinia* species. Assays with 20 nM a) Cy5EH8 or b) EH8-B were performed in triplicate with 10^7 and 10^5 copies of sonicated target genomic DNA from the species indicated above. Assay conditions are described in the text. Error bars, + standard deviations.

probes recognized EA and EC DNA less avidly, and appeared to recognize EU DNA almost as well as EH DNA (Figure 23). However the differences between the species were not significant at 10^7 copies; there appeared to be significant statistical overlap, confirming the lack of specificity of this probe. In these assays, 10^5 copies was again the lower limit of detection.

7. CPT Target Sample Preparation

7.1. Preparation of Genomic Target DNA

As detection of target organisms by CPT is based upon detection of genomic target sequence by the CPT probe, the preparation of genomic DNA must be considered in the identification and development of any useful CPT probe. Genomic DNA was prepared from *Er.herbicola*, other related test organisms (*Er. ananas*, *Er. uredovora* and *Er. carotovora*) and *E.coli* [laboratory strain TG1). Using either of the two methods, genomic DNA extracted from bacterial cells typically fragments into large 30Kb DNA segments with even larger sized DNA strands retained in the agarose gel sample wells (Figure 24). The first method used detergent and enzymatic lysis of bacterial cells followed by a protein precipitation step and a DNA ethanol precipitation step (Puregene™ method). The second method used chemical extraction of bacterial cells to release genomic DNA (phenol/guanidinium isothiocyanate) followed by ethanol precipitation of DNA (DNAzol™ method). Both methods required centrifugation steps, however the " Puregene™ " method required more procedural steps to produce an RNA-free DNA product (Table 13). An "RNase digestion" step can be added to the DNAzol method but this increased the number of additional steps and reduced the yield of DNA (Table 13). For large scale preparation of DNA, the DNAzol+RNase digestion method was used because it was more economical than the "Puregene™" method. DNA was subsequently sonicated (minimum 90 seconds - see Section 7.5 below) prior to determination of DNA concentrations either spectrophotometrically (OD_{260}) or by estimation of DNA band intensity in EB (ethidium bromide)-stained DNA agarose gels (compared with known DNA markers).

Table 13. Summary of DNA Preparation Methods

Method	Centrifugation Steps**	Incubation Steps	Sonication	Est DNA Yield (mg/ml Culture)
DNAzol™	2	1	+	0.200*
DNAzol +RNase Digestion	4	2	+	0.011
Puregene™	3	2	+	0.035
Crude Lysis (under development)	1	1	-	ND

* sample contains RNA
**initial collection of cells by centrifugation is a common step for all protocols

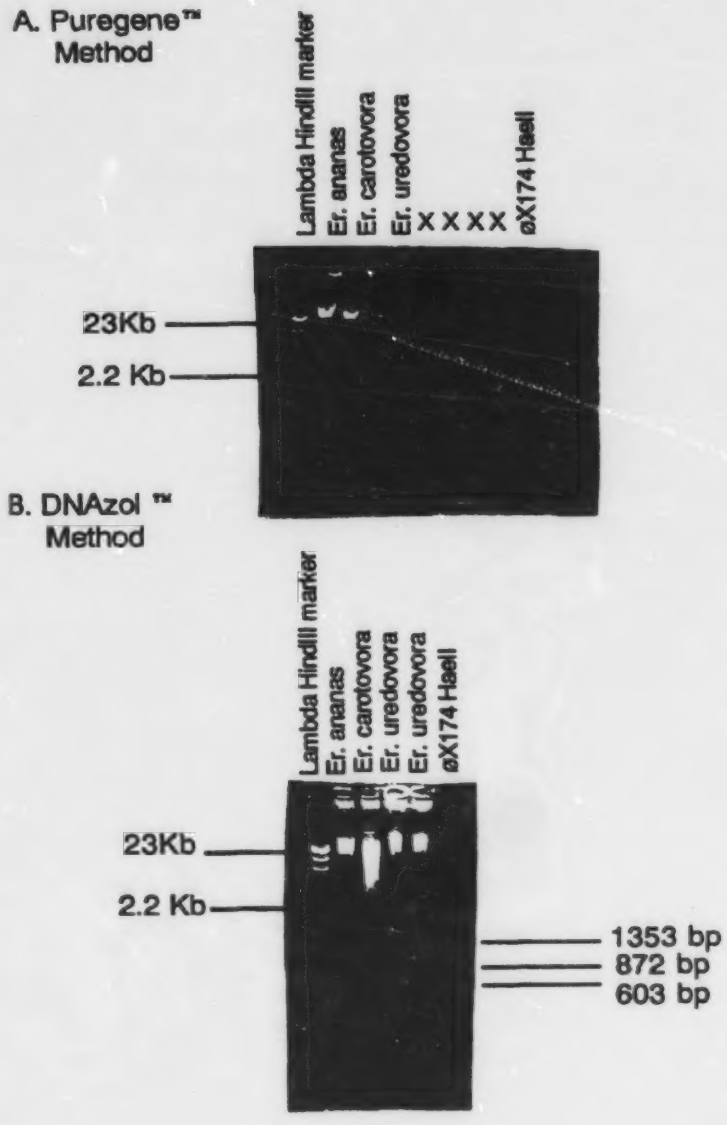


Figure 24. Evaluation of two different preparative methods for genomic DNA. DNA extracted using either the Puregene™ method [Panel A] or DNAzol™ method [Panel B] was analyzed by electrophoresis on 1% agarose gel and stained with ethidium bromide. Lambda HindIII DNA markers and øX174 HaeIII DNA markers are shown.

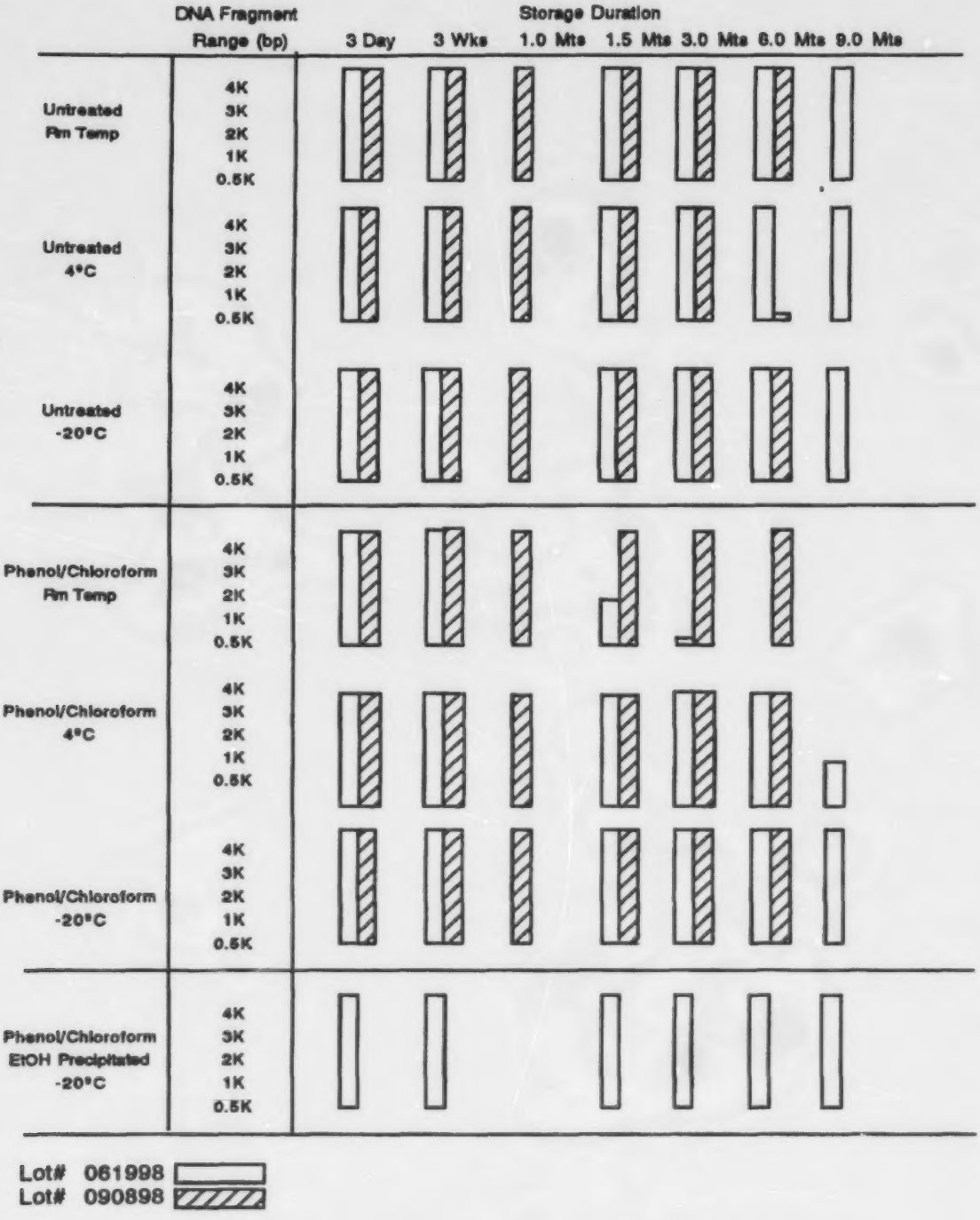


Figure 25. Stability of purified genomic DNA. Genomic DNA from *Er. ananas* was extracted using the DNAzol™ method and sonicated (Untreated). Some DNA was further purified by phenol-chloroform extraction (Phenol/Chloro-form) and a portion of this DNA was precipitated using ethanol and stored as a precipitate at -20°C (Phenol/Chloroform, EtOH Precipitated, -20°C). At various time points aliquots of DNA were taken and DNA integrity was analysed by electrophoresis on 1% agarose gels and ethidium bromide staining. The range of DNA fragments observed are represented as bar graphs above. Two preparation of *Er. ananas* DNA were tested (Lot# 061998 and Lot# 090898)

7.2. Genomic DNA isolated by DNAzol extraction is stable at ambient temperatures for up to 9 months.

As bacterial chromosomal control DNA will be used as a calibration standard for the CPT assay, it is important that the stability of purified genomic DNA preparations under various storage conditions be determined. *Er. ananas* DNA prepared by DNAzol extraction was sonicated into fragments ranging from 4Kb to 0.5Kb. DNA was stored as aqueous solutions (water only) at various temperatures [i.e. rm temp, 4°C and -20°C] (see "Untreated" in Figure 25). Some DNA was further purified by extracted with phenol/chloroform (see "Phenol/Chloroform" in Figure 25) while a third sample was stored as DNA precipitate in ethanol at -20°C (see Figure 25). At various time intervals (up to 9 months), the integrity of DNA was analyzed by electrophoresis on 1% agarose gels. Separated DNA fragments were stained with EB (ethidum bromide) and DNA fragment sizes recorded. In general, DNA preparations were stable as aqueous solutions at ambient temperatures for at least 9 months. The degradation of individual samples (i.e. "Phenol/Chloroform, Rm Temp" and " Untreated, 4°C" in Figure 25) were likely due to contamination of the DNA sample during sampling for analysis.

7.3 . Current chemical extraction methods are inadequate for isolation of DNA from FT (field trial) samples.

DNA from *Er. herbicola* and *E.coli* were previously prepared by DNAzol extraction, sheared to 30Kb DNA fragments by passage through 22 gauge syringe needles and ethanol-precipitated (Figure 26). RNA is visible as stained material ranging from 310 bp to 1353 bp. DNA was extracted from a field trial sample which contained *Er herbicola.* Field Trial 33, Sample 35 (a 1 ml aqueous solution) was divided into 0.75 ml and 0.25 ml aliquots and centrifuged at 13K Xg for 10 minutes. Pelleted material (not detectable visually) was extracted with 100 µl of DNAzol and ethanol-precipitated. Each of the pelleted material was washed twice with 80% ethanol , dried and resuspended into 10 µl of sterile water. From 10 µl of DNA extract, 4 µl was used for spectrophotometric analysis [diluted in 1 ml and measured at OD_{260}], and 4 µl was used for analysis by agarose gel electrophoresis. DNA was not detectable spectrophotometrically or by EB-staining after separation of DNA by agarose gel electrophoresis. Even after twenty-fold concentration (20X) of the Field Trial DNA samples (4 µl DNA in 50 µl vs. 4 µl of DNA in 1 ml of sterile water) no DNA was detectable spectrophotometrically (OD_{260}).

In the preparation of genomic DNA samples using DNAzol extraction and ethanol precipitation, results indicated that DNA yields could not be reliably obtained from 1 ml bacterial culture of *Er.*

herbicola at less than 10^6 CFU/ml. Further work to determine if DNA can be extracted from field trial samples, or samples containing smaller cell numbers (< 10^6), was halted until a more efficient method of extracting DNA from small numbers of bacterial cells could be developed.

7.4 . Development of CPT lysis buffer for preparation of Erwinia DNA.

To develop a "CPT-compatible" lysis buffer for genomic samples, we investigated a number of proteinase combinations. We chose a basic STMT buffer (0.1 M NaCl, 10 mM tris pH 8.0, 10 mM $MgCl_2$, 5% tritonX-100) supplemented with 1 mg/ml lysozyme and 1 mg/ml lyphostatin. DNA incubated in this buffer did not degrade even after 2 hours incubation at room temperature (only 30 Kb DNA fragments and EB-stained material retained in gel sample wells were visible). Degradation (smearing of EB-stained DNA fragments < 30Kb) material was observed only after 24 hours of incubation at room temperature (data not shown).

We hypothesized that an addition of a protease (i.e. Proteinase K) might help to degrade scaffolding proteins which may be coating and protecting genomic DNA from endogenous nucleases. The buffer-enzyme mixture (STMT supplement with lysozyme and lyphostatin) used to prepared crude DNA lysate from bacterial cell pellets was supplemented with Proteinase K or achromopeptidase. The effect of proteinase K at (0.1 mg/mL) significantly increased the amount of the 30Kb DNA fragment but only marginally increased amount of degraded DNA (Figure 27, Panel A). The presence of the 30Kb DNA fragment in all proteinase K-treated and untreated samples at all time points, indicates poor degradation of DNA. Similar results were obtained when achromopeptidase was used in place of proteinase K (Figure 27, Panel B). Since DNA prepared in both of these experiments were purified by phenol-chloroform extraction and ethanol-precipitation (prior analysis by agarose gel electrophoresis) genetic material typically trapped in the agarose gel sample wells is absent.

7.5 . Physical shearing of bacterial genomic DNA

Genomic DNA for *Er. herbicola*, *Er. uredovora*, *Er. carotovora*, and *Er. ananas* was prepared by the DNAzol™ method and subjected to various shearing techniques. Shearing by repeated passage through syringe needles (22-26 gauge) gave limited success, generating predominantly DNA fragments of 30Kb in length. Sonication was require to efficiently generate smaller DNA fragment sizes. Sonication for 90 seconds produced DNA fragments ranging from 300 bp to 2500 bp (Figure 28). Increasing sonication times up to 180 seconds did not produce significantly smaller DNA fragments.

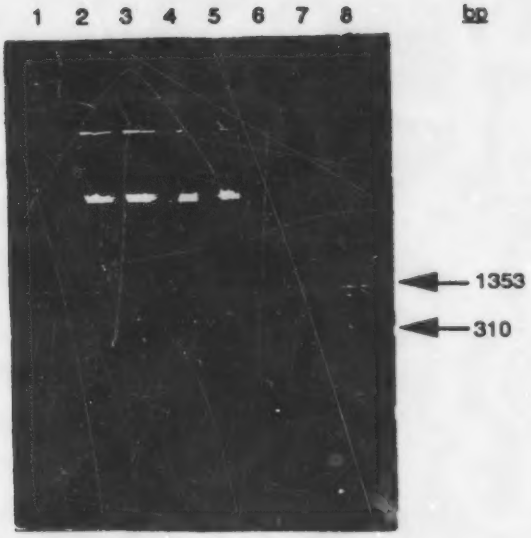


Figure 26. Preparation of *Er. herbicola* field trial samples. Chromosomal DNA samples were prepared by extraction with DNAzol™ (Canadian Life Technologies) and ethanol precipitation. Samples were separated by electrophoresis on 0.7% agarose and stained with 1 µg/ml ethidium bromide. Lane 1. øX174 HaeIII marker (0.16 ug); Lane 2. *Erwinia herbicola* chromosomal DNA Lot#1103A (0.5 ug); Lane 3. *Erwinia herbicola* chromosomal DNA Lot#1103A (0.5 ug); Lane 4. *E. coli* TG1 chromosomal DNA Lot 1126 (0.5 ug); Lane 5. *E. coli* TG1 chromosomal DNA Lot 1126 (0.5 ug); Lane 6. Field Trial 33, sample 35 Lot#1217A (? ug) - 0.75 ml extract; Lane 7. Field Trial 33, sample 35 Lot#1217B(? ug) -0.25 ml extract; and Lane 8. øX174 HaeIII marker (0.16 ug).

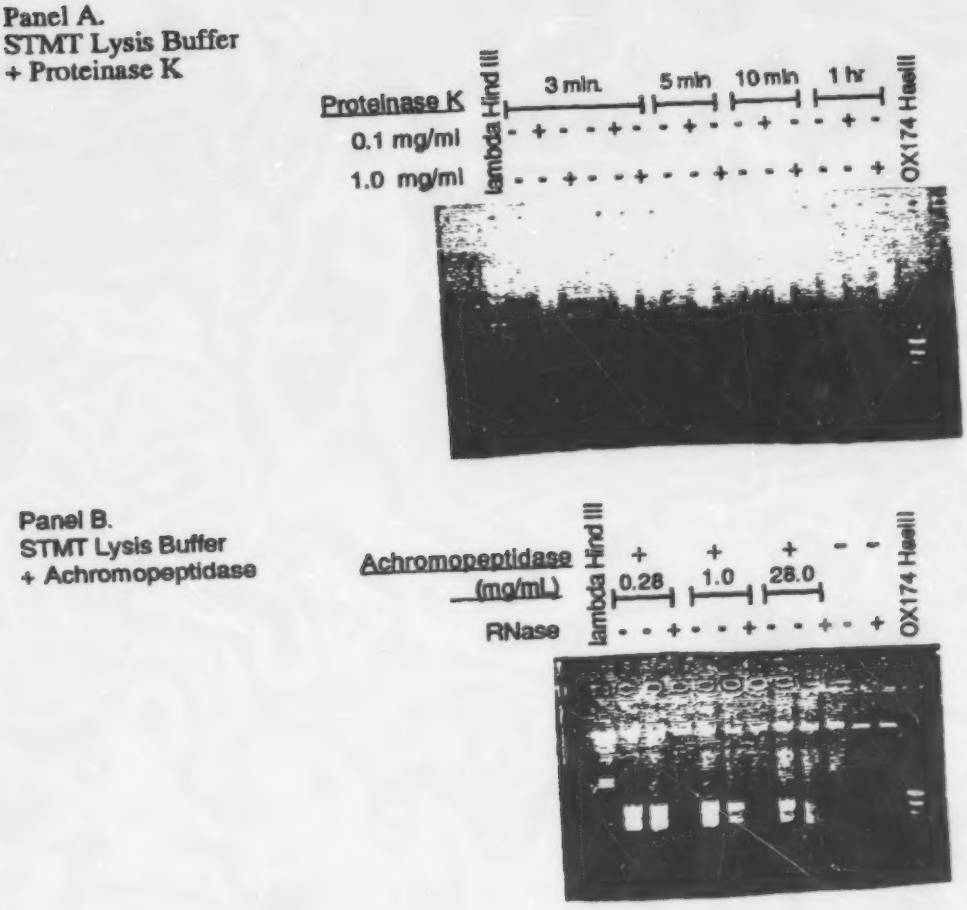


Figure 27. Evaluation of different enzymatic preparative methods for crude genomic DNA. One and one-half milliliters (1.5 ml) of bacterial cells were collected from overnight culture of *Er. herbicola* (OD600=0.8-1.0) by centrifugation. Cell pellets were resuspended in STMT buffer supplemented with final concentrations of 100 ug/ml lysozyme and 3 ut/ml lyphostaphin. In Panel A, Proteinase K was added to a final concentration of 100 ug/ml or 1 mg/ml, and samples were incubated at room temperature for various time intervals from 3 minutes to 1 hour (Panel A). In Panel B, cell pellets were resuspended in STMT buffer containing various concentration of achromopeptidese (final concentrations of 0.28 mg/mL, 1.0 mg/mL or 28 mg/ml) and samples were incubated at ambient temperature for 5 minutes. Certain samples were digested RNAse (final concentration = 0.25 mg/ml) at 37°C for 15 minutes. Samples not RNAse digested are shown in duplicate (Panel B). Products from both experiments were analyzed by electrophoresis on 1% agarose gels and staining with ethidium bromide. Also shown are DNA fragment size markers, Lambda Hind III and ØX174 HaeIII.

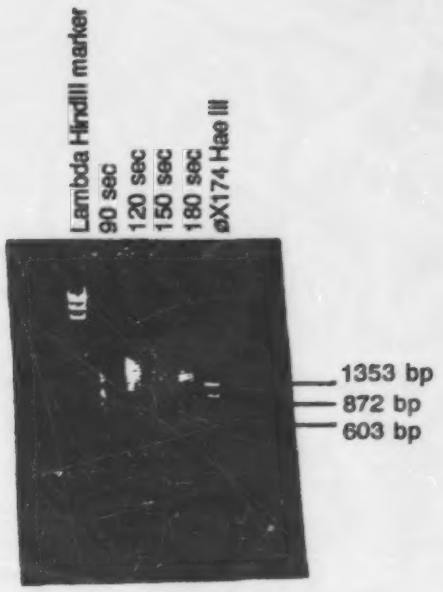


Figure 28. Sonication of *Erwinia uredovora* Genomic DNA. Bacterial DNA was prepared using the DNAzol™ extraction method and sonicated for various time periods (lanes marked at 90 sec, 120 sec, 150 sec and 180 sec) and separated by 1% agarose gel electrophoresis. Included in the gel are lanes showing the lambda HindIII DNA marker and øX174 HaeIII DNA markers.

Discussion

1. Probe Design Considerations

Design of suitable probes for *Erwinia herbicola* was critical to ensure maximum specificity for the target. Although it was still necessary to test the probes in CPT assays, the OLIGO primer analysis program was useful in predicting the behavior of synthesized CPT probes. For example, EH6 probe had exhibited a large degree of C2 activity in CPT assays with synthetic target. In this case the probe was predicted to form a very stable 5' hairpin with a Tm of 64 °C, greater than the reaction temperature. Although the A(4) region was not involved in its formation, it was immediately downstream of the hairpin, hence RNaseH could theoretically still bind upstream to the site and exact cleavage of the probe. *E. coli* RNaseH is known to cleave single stranded RNA immediately 3' of a RNA-DNA duplex (Lima and Crooke 1997), hence this may be a possible explanation for the high C2 noted for EH6, and also for EH10 during probe screening with synthetic target.

From the genes surveyed, some important observations were made regarding suitability of genes as potential CPT targets. The 16S rRNA sequences would likely make poor CPT targets for any bacterial species. As 16S rRNA is highly conserved among bacteria (Harada and Ishikawa 1997), any CPT probes derived from these sequences would likely not be species-specific. Gene sequences present on plasmids (such as the pPATH plasmid in *Er. herbicola pv. gysophiliae*) may be useful in distinguishing pathogenic from non-pathogenic strains, but cannot be relied on to detect all *Er. herbicola* species. This was reflected by the lack of reactivity of EH5 and EH6 probes in isotopic assays with this particular strain of genomic target. Both probes had been derived from the dimethylallyltransferase gene carried on the pPATH plasmid, which, from lack of reactivity of the probe, was not present in this particular target strain.

1.1. Limitations of the Database Approach to CPT Probe Design

The major drawback of using genetic databases (Genbank, EMBL) to design CPT probes, is that the information present in the database is necessarily limited to the sequence submissions made to the databases. Complete genome sequences are available for only a few organisms (currently *E. coli*, *Rickettsia prowasekii*, *Mycobacterium tuberculosis*, *Mycoplasma pneumoniae* and *genitalium*, *Helicobacter pylori*), the yeast *Saccharomyces cerevisiae*, and some bacteriophages. An obvious point is that by limiting CPT probes to previously sequenced genes only, more target-specific sequences are likely to be missed. Also, even though searches through the database may suggest a

particular CPT probe will be specific for a target organism, the suggestions are by no means definitive due to the lack of comprehensive sequence information available. Therefore, actual CPT assays may reveal unexpected cross-reactivity of the probe to heterologous targets. This strongly suggests that another experimentally-based approach may be required to supplement probe design.

Such cross-reactivity as described above was observed for EH8 during screening against heterologous target. The database approach predicted reasonable specificity of EH8 for its intended target (tyrosine phenol-lyase), with 5 mismatches in the 28mer probe sequence to the closest matching sequence (*E. coli* pyruvate kinase gene). However, this probe showed a strong and unexpected cross-reactivity to *E. coli* non-specific target as well as to the other *Erwinia* species tested. From theoretical considerations, 5 mismatches (20% variability to the target sequence; 21 degree difference in Tm) should have ensured adequate specificity, especially since one mismatch occurred in the ribo region. However in practice, such specificity was not observed. Enough sequence identity existed on either side of the ribo region to presumably cause a clamping effect, especially since the sequences were GC-rich on either side (Figure 27). Experiments modifying concentrations of EDTA or spermine, or increasing temperature, failed to relieve the cross-reactivity to *E. coli.* and by extension other *Erwinia* species (although the other species were not extensively tested). The *Er. herbicola* pyruvate kinase sequence is currently not available in the Genbank and EMBL databases (March 1999), but may be expected, from these results, to share significant sequence identity to the *E. coli* sequence. Theoretical analyses in OLIGO suggested that decreasing the probe length to 20 from 28 should increase specificity for probe hybridization to *Er. herbicola* DNA (Table 13), but this remains to be tested empirically. A similar lack of specificity was seen for CPT probes previously designed for *Bacillus subtilis* ; although designed from the division initiation gene from *B. subtilis*, the probes detected all *Bacillus* species tested (ID Biomedical, 1996). These results combined suggest that an experimental approach to CPT probe design will be far more advantageous than relying on a database approach to obtain target-specific probes. Such an experimental approach would be expected to require extensive workup to obtain a species-specific probe.

An experimental approach to CPT probe design would likely be more appropriate for cases where genomic sequences for target organisms are incomplete, where confounding non-target organisms are very closely related genetically to the target organism, or where specificity cannot be determined by a presence/absence" phenomenon such as for antibiotic resistance genes or unique repeat sequences. The best experimental design for CPT probes would likely use a subtractive

Figure 29. Comparison of EH8 with *E. coli* pyruvate kinase sequence. The ribo region is underlined. Sequence identity is indicated by hatches.

```
EH8:     ACGCGGGGCGGAAAACCTGCTCTCGCAG
         ||  |  ||||||  |||  ||||||  |||||
ECOPK1:  ACTCAGGGCGGTAAATCTGCTCGCGCAG
```

Table 14. Comparison of Theoretical Tms and Mismatches for Abbreviated EH8 Probes

Oligo Length [1]	# Mismatches to ECOPK1	Approx. Theoretical Tm, °C [2]	Mismatch Tm, °C [2]
28	5	91.1	69.7
20	4	72.8	48.8
18	3	67.0	47.0
16	2	60.7	45.7

Notes: [1] Lengths are calculated by removing equal numbers of nucleotides from either end, and encompass the ribo region.

[2] Calculation in OLIGO program based on the nearest neighbor method for DNA

hybridization approach, where the genomes from non-target organisms are hybridized with the genome of the target organism; common sequences between the species are removed and anything left over is amplified, cloned, sequenced and analyzed for specificity. CPT probe sequences may then be derived from the cloned sequences and re-analyzed against specific and non-specific targets. A PCR-amplified subtractive hybridization approach was able to identify strain-specific sequence differences within the species *Helicobacter pylori* (Akopyants *et al.* 1998). A related technique, representational difference analysis, was used to identify genomic differences between pathogenic strains of *Vibrio cholerae* (Calia *et al.* 1998). The subtractive hybridization approach to CPT probe design may thus be better suited for bacteria like *B. globii* or *Er. herbicola*, that are very closely related to other species. As only a few dozen gene sequences are available for *Er. herbicola* in Genbank and EMBL, and *groE* gene sequence analysis suggests a very close genetic relationship between *Er. herbicola* and the non-specific target *Er. ananas* (Harada and Ishikawa 1997), an experimental approach should thus be a more successful probe design strategy.

With respect to product detection, probe design was also important for a non-gel system to enhance the separation of labelled intact and product species. Oligonucleotides of any length carry a constant charge/mass (e/m) ratio because of the constant number of charges per base. Hence, separation by CE in a non-gel system is dependent on modifying the e/m ratio between the intact and cleaved probe products. Conventional gel systems exert a sieving effect such that separation becomes independent of the charge and dependent only on the length of the oligo. To ensure separation between intact and cleaved probe species for CE detection in a non-gel system, the 3' end required modification as by convention, the 5' end carries the fluoro/chromophore. A neutral or positively-charged tag on the 3' end exerts a "drag" on the intact probe compared to the cleaved probe, thereby changing the e/m ratio of the intact probe and providing better resolution. Biotin, a neutrally-charged molecule that is commonly used as an oligo tag, appears to be an effective e/m modifier and does not appreciably interfere with CPT assays. Although three biotins provided the best resolution between intact and cleaved probe, one biotin was sufficient to result in adequate resolution in a non-gel system. This was noted in experiments on-chip at the University of Alberta, but not addressed here. Other molecules, such as digoxigenin, will exert the same effect.

2. Development of Optimal CPT Conditions

We attempted to optimize the CPT assay to maximize sensitivity and specificity towards *Er. herbicola* target. This required a 2-step approach: optimizing the CPT assay conditions itself, and adapting the assay for CE-LIF on a microchip.

2.1. Optimization of CPT Assay

The presence of non-homologous DNA in a CPT reaction affects both the background and the extent of probe cutting, thereby reducing reaction sensitivity (Modrusan *et al.* 1998). Low levels of extraneous DNA result in a high background, while high levels of DNA inhibit the reaction through non-specific interactions with both RNaseH and probe. These effects were observed by using unsonicated target DNA and high-copy-number DNA samples in both fluorescent and radioactive assays. Addition of spermine to the CPT reaction reduces non-specific DNA-probe interactions and inhibits binding of RNaseH to target DNA in the absence of probe (Modrusan *et al.* 1998), thereby increasing the sensitivity and specificity of the probe. This effect was noted to some extent in isotopic assays, although at high concentrations spermine inhibited probe cutting. Increasing the amount of RNaseH added also increased the turnover rate of the reaction, thereby increasing assay sensitivity.

The original CPT assays involved starting the reaction from ice, then putting the tubes in a block heater at the CPT reaction temperature. In experiments performed at ID Biomedical, pre-heating the tubes to the reaction temperature, prior to adding cocktail, reduced the temperature transition time and prevented non-specific probe binding to heterologous DNA and cleavage during the temperature transition time. Conversely, stopping the reaction while at the reaction temperature results in improved specificity for the same reason (F. Bekkaoui, personal communication). However, in our experiments with EH8 and *E.coli* DNA increased specificity was not observed (Figure 13), likely due to the peculiarity of the probe sequence as explained above.

To try to determine why EH8 showed a lack of specificity even at different conditions which would normally increase specificity, a more detailed comparison of the sequence with its likely complement from *E. coli* pyruvate kinase, was carried out in the OLIGO program. The lack of specificity was postulated to be due to the GC-rich regions on either side of EH8 acting to "clamp" the probe to *E.coli* DNA (Figure 30). This clamping effect persisted despite assay modifications designed to increase binding specificity, as addressed in the Results section.

2.2. Adapting CPT Assay to CE-LIF

The original CPT assay was developed in TES buffer. This buffer was adequate for the detection conditions originally used, *i.e.* conventional gel electrophoresis with autoradiography or phosphor imaging. However the buffer proved inadequate for CE-LIF of CPT assays. The buffer had a

very high conductivity relative to that of the sample, thereby reducing or preventing sample loading by electrokinetic injection; this was readily observed by comparing the detection of another CPT probe, mecA301-1, dissolved in water with probe dissolved in TES buffer, other CE conditions being equal (Figure 17).

Phosphate buffer was assessed for its utility as a dual purpose buffer for CPT assay and CE separation on microchip (see below). Phosphate buffers of varying ionic strengths and pH are very commonly used as background electrolytes in CE. ID Biomedical had already determined that 20 mM phosphate pH 6.8 was effective in CPT assays (F. Bekkaoui, personal communication); this concentration and pH were consistent with other CE applications using phosphate buffer that are described in the literature. Phosphate buffer gave better results than TES buffer even in the probe screening with ^{32}P, hence all CPT assays were performed in 10 mM phosphate pH 7.0.

Stopping the CPT reaction was required as earlier fluorescent experiments with EH3 probe showed increasing C2 activity in samples at room temperature. EDTA (50 mM pH 8.0) was used. Studies looking at different EDTA concentrations suggested that a concentration as low as 10 mM would be sufficient to stop the reaction.

We did not try other modifications to the assay, such as changing salt concentrations or adding formide to alter hybridization stringency, as the effects on the enzyme were not precisely known (F. Bekkaoui, personal communication). However, any modification to the CPT assay must also be considered in light of adjusting capillary electrophoresis conditions for detection of cleaved products. For example, changing the salt concentration has a significant modifying effect on electroosmotic flow during capillary electrophoresis (Kuhr 1993), as does adding organic solvents (van de Goor *et al.* 1997). Therefore it is prudent to keep the reaction conditions as simple as possible to maintain a contiguous buffer system for both assay and detection.

3. CE-LIF Detection Considerations

In this work, the optimization of detection of CPT products by CE-LIF necessitated a change of buffers and run conditions for both the CPT assay and CE-LIF detection on microchip. One major stumbling block was the use of TES buffer in the CPT assay; it was found to have too high a conductivity to allow sample to be electrokinetically injected into the column for consistent and sensitive detection. The voltage required to perform CE, using TES buffer as the carrying ion, was too high to be useful for the microchip (T. Tang, personal communication). As TES buffer

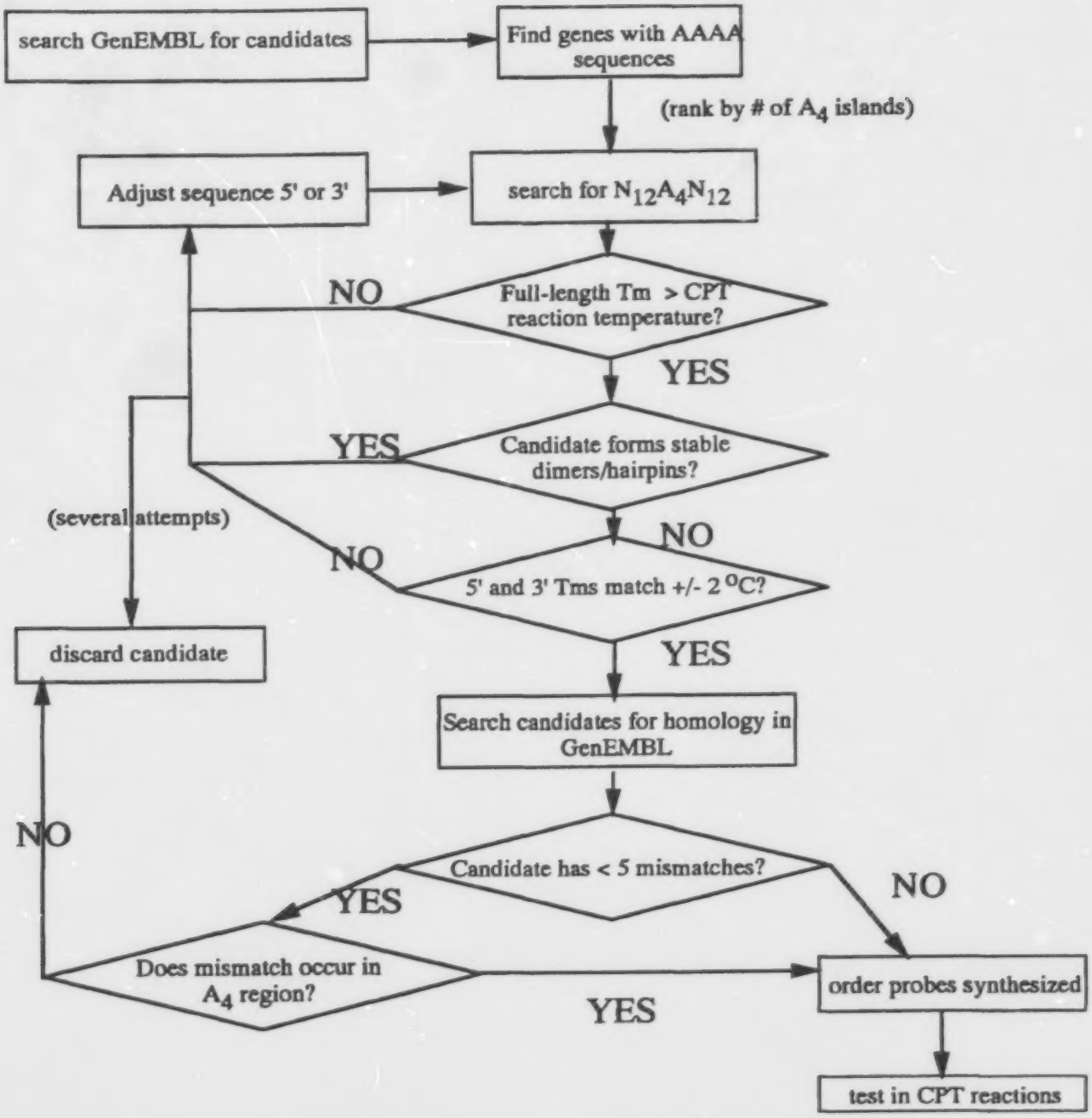


Figure 30. Generation of *Er. herbicola* CPT Probe Sequences. Probes were derived from the genetic sequences of *Er. herbicola* using this flowchart, as described in the text.

was not ideal for CE-LIF, we needed to find a buffer compatible with both CPT and CE-LIF detection, in order to use the same buffer for both the assay and for identification. Phosphate buffers at a range of ionic strengths and pH are common ion carriers in CE . Also, phosphate buffers at near-neutral pH and low ionic strength (10-20 mM) are compatible with CPT assays; tests by ID Biomedical showed that 20 mM phosphate pH 6.8 was equivalent to 20 mM TES pH 6.8 in CPT assays. Hence we decided to continue development of the assay in phosphate buffer. Ten mM sodium phosphate buffer at pH 7.0 proved to be slightly better than 20 mM pH 6.8 in isotopic CPT assays, hence that buffer was chosen as the base electrolyte.

4. CPT Assay Sensitivity and Reproducibility Issues

The sensitivity of the fluorescent CPT assay with CE detection, was hoped to reach that of the isotopic assay, *i.e.* on the levels of thousands of copies in a sample. A reliable estimate for sensitivity was determined to be on the order of 10^5 copies of DNA. This result was consistent with those obtained by ID Biomedical for different detection formats (unpublished data). One limit to assay sensitivity using a benchtop CE was the use of gel-filled columns; electrokinetic injection is the only sample injection available, although it is not the most sensitive or quantitative method. However, hydrodyamic or pressure injection would extrude the gel from the column. Electrokinetic injection is also the only injection method available on the microchip. Studies injecting probe in different components of assay cocktail, showed that the cocktail components decreased the amount of probe loaded onto the column by up to 90% (peak height) compared to injection from water, likely due to discontinuous buffer systems used in the gel column (TRIS/borate/urea vs. phosphate) and analyte competition with background electrolyte in the CPT assay (not shown). Hence a contigous assay/separation buffer system may assist in overcoming this obstacle.

A working detection limit of 10^5 copies of chromosomal DNA may not be sensitive enough for field detection of some virulent organisms, depending on the efficiency of sample collection and the natural virulence of the target organism itself. To obtain a detection limit of 10^5 copies (50 pg/μL), and assuming that one chromosomal copy equals one live infectious organism or CFU/PFU, current collection techniques would require concentration of air or water samples to an equivalent concentration of 10^{10} CFU/ml. In contrast, virulent organisms such as *B. anthracis* require only a few thousand copies to produce illness; other organisms of military significance, such as smallpox virus or *F. tularensis*, require less than 100 organisms to cause disease in a

susceptible host (Franz *et al.* 1997). Current PCR-based approaches can detect organisms at a level two orders of magnitude less than the current CPT assay at this point. Sample size and concentration is thus an important consideration when working with a linear detection method as opposed to an exponential detection method.

One original goal of this part of the contract was to reduce the assay incubation time required to a minimum, while maintaining peak assay sensitivity. This goal was not accomplished. In light of the low assay sensitivity with CE-LIF detection, reducing the assay time from 30 minutes would compromise the sensitivity of target detection further under the current conditions. One method to circumvent this would be to increase enzyme concentration in the samples; this might interfere with sample separation in a non-gel system, as the presence of extra enzyme might theoretically slow the migration of the probe.

The reproducibility problems of CPT assays with fluorescent probe suggested that the assay-to-assay variability was inherent in part to electrokinetic injection of samples onto the column. Electrokinetic injection depends on the charge of the analytes and the ionic composition of the analyte solution; analytes compete with buffer components for sample loading during voltage injection. This was seen with assay samples incorporating internal standards, which showed as much as 20% variability of the internal standard peak areas between different samples. The variability appeared greatest in samples containing very high or very low genomic target copy numbers.

A second reproducibility problem may have been due to the lack of sample denaturation prior to assay and before CE analysis. This was more applicable to genomic targets than to synthetic targets. Occasional column plugging problems were noted in practice, especially with high genomic DNA. However, isotopic assays suggested that sensitivity of the assay would not be compromised when genomic samples were not denatured prior to assay. Denaturation of genomic samples was not performed with CE-LIF analysis for two reasons. Pure target samples were sonicated and supplied in distilled water, which destabilizes double-stranded DNA. Secondly, sample denaturation prior to analysis was found to degrade the fluorescent signal for Cy5. As the goal is to simplify the assay as much as possible for the microchip format, denaturation steps would likely impose more design constraints on the prototype device, such as requirements for a variable heat source, heat-stable chip coatings and insulation.

Of note, another potential barrier to CPT as a general BW identification method, is that several viruses of potential military significance, such as the filoviruses (Ebola and Marburg hemorrhagic fevers) contain RNA genomes as opposed to DNA genomes. Currently, CPT is designed for detection of only DNA genomes. The development of RT-CPT (F. Bekkaoui, personal communication), analogous to RT-PCR, may overcome this potential difficulty, although this technique will require another enzyme that is maximally effective at a lower temperature than that of the CPT assay (42 °C for reverse transcriptase) and thus likely may present some further design difficulties in adapting to microchip format.

5. Genomic DNA Preparation Issues

Chemical and mechanical extraction methods (DNAzol and sonication) could not be used for preparation of genomic DNA from small numbers of bacteria (i.e. field samples). Losses due to adhesion to multiple container surfaces, extraction and precipitation procedures were unacceptable. The ideal DNA extraction method to prepare field trial samples should be simple, fast (requiring only 5 minute incubation at ambient temperature) and use only a single lysis buffer (which is compatible with the CPT assay). Work was done to develop a simple lysis buffer that would adequately prepare bacterial genomic DNA as a CPT template. The basic buffer for this work was STMT buffer supplemented with lysozyme and lyphostatin. DNA was observed to remain intact even after 2 hours incubation at room temperature. Degradation was observed only after 24 hours of incubation at room temperature. Although these peptidylglycanolytic enzymes can disrupt bacterial cell walls, endogenous nucleases required at least 24 hours in order to adequately digest and release genomic DNA, far too long to be useful in the field.

Genomic DNA extracted from bacterial cells exist as long molecules which produce a very viscous solution. Separation of bacterial genomic DNA by agarose gel electrophoresis and ethidium bromide staining revealed that the genomic DNA naturally shears to 35 kb DNA fragments. Much larger unsheared DNA was retained in the sample wells because they were too large to resolve in 1% agarose. The DNA concentration of this DNA solution was thus difficult to determine because it consisted of "pockets" of heterogenous DNA concentrations. These pockets made it impossible to accurately take samples by micropipettors. The CPT assay works optimally with genomic target DNA sheared to an average DNA fragment size of 1 to 2 Kb (F. Bekkaoui, ID Biomedical, personal communication). DNA from *Er. herbicola* and *E. coli* can be sheared by repeated passages through 26 gauge syringe needles, however, shearing by sonication is much faster and more efficient.

Simple disruption of bacterial cells is clearly not enough to prepare bacterial DNA for assay by CPT. Bacterial DNA is packaged in scaffold or nuclear-matrix proteins which may interfere with probe hybridization by masking target sequence. An effective lysis procedure must be able to release the DNA from protein complexes (genetic material trapped in agarose gel well slots) and allow intrinsic nuclease to degrade DNA into fragments of adequate size for CPT. The current buffers tested did not adequately release DNA from protein complexes or allow intrinsic nucleases to sufficiently degrade the the chromosomal DNA within 5 minutes of incubation at ambient temperature (22°C). The addition of proteinase K and achromopeptidease was intended to allow the release of DNA from protein complexes; however these enzymes may have the adverse of effect of destroying intrinsic nucleases. Future development of an effective bacterial CPT lysis buffer may thus require additional supplementation with DNAses or endonucleases. Including these enzymes, in proper balance with proteases added, should allow chromosomal DNA to be released from the protein complexes and the DNA to be digested by nucleases into adequate sized fragments for CPT assay, before the nucleases are degraded.

6. Summary: Final Assessment of CPT Assay Adaptation to Microchip Format

The CPT assay is a simple, isothermal method of detection of DNA that does not rely on exponential amplification of signal. However, despite its theoretical ease of use, it proved challenging at several levels to adapt the assay to a rapid microchip format for field trial use. Success of the assay depends in large part on obtaining a target-specific probe. Probe design was problematic as long as a) the design was dependent on database information and b) the target sequences were not unique to the target species or strain. Extensive workup was required to establish the characteristics of the probe designed using information from a database. The linear reaction kinetics of probe hybridization and probe turnover suggested that sensitivity of the assay depended to a great extent on the sensitivity of the detection method used. Under current conditions it was difficult to reduce the length of time to perform the assay while maintaining high sensitivity, even with a highly specific probe. Its relatively low sensitivity with benchtop CE-LIF detection in gel columns (10^5 copies of chromosomal DNA) showed that although this assay may be useful in a clinical diagnosis setting, where sample is relatively concentrated and easily available, its utility in the field may be less so when very dilute solutions of extremely virulent organisms are concerned. Its wide assay-to-assay variability under current conditions suggested that it was not able to provide an absolute quantitative answer. This assay was able to provide a yes/no answer, and with appropriate calibration, should be able to provide a relative estimate of the amount of target present in a sample. In conclusion, much more workup is required to establish the assay to reach the goals

outlined for full adaptation to microchip format (rapidity, high sensitivity and specificity) for field trial use, although significant strides have been made to bring the assay to this point.

Methods and Materials

1. Design of CPT Probes from *Er. herbicola*

Probes were designed as shown by the flowchart in Figure 30. Potential CPT gene targets were listed by identifying the presence of A_4 islands (sequences with 4 deoxyadenosine residues) within the known genes of *Er. herbicola*. To obtain the sequences, a MEDLINE search (http://www.ncbi.nlm.nih.gov/PubMed) was performed in the GenBank gene sequence database (National Centre for Biotechnology Information, National Institutes of Health, Bethesda, MD) for *Er. herbicola* genes. The sequences were downloaded from the database and pasted into the OLIGO Primer Analysis Version 5.1 program for Macintosh computers (NBI/Genovus Inc., Plymouth MN). Each gene was surveyed for the presence of adjacent A_4 islands, and frequencies of A_4 islands calculated using the formula F = (# A_4 islands) / (sequence length) x 1000.

For each gene with a high frequency of A_4 islands, a 28-30mer sequence flanking either side of the A_4 island was analyzed for secondary structure formation (dimers or hairpin structures) using the OLIGO program. A candidate was acceptable if a), the energy of formation (ΔG) of the secondary structure was greater than zero at the reaction temperature (taken to be 60 °C); b), the melting temperature (Tm) of the secondary structure was lower than the CPT reaction temperature; and c), the A_4 island was not involved in secondary structure formation. These parameters were to ensure that secondary structures would not contribute to high C2 activity.

The Tm of the entire probe sequence was calculated using 2 separate methods: the Wallace rule (Tm = 2 x AT + 4 x GC) and the salt-concentration method (salt = 50 mM, formamide concentration = 0%, DNA concentration = 200 nM) outlined in the OLIGO program. A sequence was deemed acceptable if the Tm was greater than 60 °C using both Tm methods; this would ensure that the probe would remain hybridized to the template at the CPT reaction temperature.

The Tms of the sequences 5' and 3' to the A_4 island were calculated and compared. For a sequence to be considered acceptable as a CPT probe candidate, the 5'Tm and the 3'Tm ideally were comparable within 2 °C, to ensure that the cleaved products would melt off the template at about the same time. If the 5' and 3' Tms were not comparable, the sequence was adjusted by either adding nucleotides or shifting the sequence 5' or 3' as needed until the Tms became comparable.

Candidate probe sequences were then tested for specificity by comparing them to the known gene sequences in the Genbank and EMBL databases, using the FINDPATTERNS utility in the GCG Wisconsin package and accepting up to 7 mismatches. An acceptable candidate sequence was deemed unlikely to cross-react if it had a minimum of 5 mismatches (less than 20%) within the sequence. Acceptable 5'-fluorescein-labelled probes or Cy5-EH8 were then obtained from Integrated DNA Technologies (Coralville, IA).

2. Characterization of CPT Probes by UV and LIF-CE

Probes were resuspended to a concentration of 200 μM in sterile distilled water. For UV-CE, probes were diluted to 20 μM in sterile distilled water and 10 μL of sample was run on a Beckman eCAP100ssDNA CE gel column as described below. For LIF-CE, probes were diluted to 1, 10 and 100 nM in distilled water and 10 μL samples were run as described below.

The CE columns used were Beckman eCAP100ssDNA gel columns, total column length=27-47 cm, length to detector=20-40 cm, i.d.=100 μm. The running buffer was Beckman CE column buffer (44% TRIS / 56% borate / 7M urea pH 8.3). Buffers were filtered through a 0.22 μm filter and degassed under vacuum for 15-20 minutes prior to use.

All CE was performed on a Beckman P/ACE System 5010 CE unit with on-column UV or LIF detectors. For UV, detection wavelength was set to 254 nm. For fluorescein detection by LIF, laser energy was supplied by a Beckman P/ACE System Laser Module 488 unit with a 3 mV Ar laser. Fluorescein emission was collected by 2 optical filters at right angles to the excitation beam (excitation wavelength 488 nm, emission wavelength 520 nm). For Cy-5 detection by LIF, laser energy was supplied by a Beckman 635 diode laser module (excitation and bandpass filters 635 nm; detection at 650 nm). All samples were run at 30°C unless otherwise specified. Samples were electrokinetically injected onto the column as described for various protocols. Samples were separated at various voltages and times as described in the Results section, typically at a field strength of 250-300 V/cm. UV-CE samples were typically run in reverse polarity mode (l.d.=20 cm) and LIF-CE samples were run in normal polarity mode (l.d.=7 cm) unless otherwise specified.

Beckman P/ACE System 5000 Series Station software for Windows was used for data collection, analysis and instrument control. Signals were measured in absorbance units (AU-UV) or relative fluorescence units (RFU-LIF) with a data collection rate of 5 Hz. For data analysis, retention

times (Rt) in minutes of each detectable peak (s/n ≥ 2) was determined. Peak areas were calculated for detectable peaks and expressed as percentages of the total area.

3. 3'Radiolabelling of CPT Probes

Two picomoles of CPT probes were 3'radiolabelled with 25 μCi (alpha)^{32}P-ddATP (Amersham, Mississauga ON) and 48 units of calf thymus terminal deoxyribonucleotidyl transferase (TdT; Pharmacia, Laval PQ), at 37 °C for 15 minutes. The labelling reaction was stopped by adding 8 μL of 0.5 M EDTA, and diluted to 80 μL with distilled water. Probes were purified on NICK Sephadex G-50 oligonucleotide separation columns (Pharmacia, Laval PQ) in 400 μL of distilled water, resulting in a final concentration of 5 nM. 10x SSC was added to a final concentration of 0.1 x SSC. Purity of probes was checked, before and after NICK purification, on a 20% polyacrylamide/7 M urea gel, and gels were exposed overnight on XAR-5 film (Kodak) to obtain autoradiograms.

4. Preparation of Synthetic Target

Synthetic target (complementary to the probe sequence) was synthesized at DRES on a Beckman Oligo 1000 DNA synthesizer, using standard Beckman reagents and protocols supplied by the manufacturer. Target concentrations were adjusted to 100-200 μM in sterile distilled water. Purity of the target was assessed by UV-CE as described above for CPT probes. Targets were used as synthesized with no further purification for probe screening.

5. Preparation of Genomic Target

Erwinia herbicola (ATCC # 33243), *Erwinia ananas* (ATCC # 33244), *Erwinia carotovora* (ATCC # 138) and *Erwinia ureodovora* (ATCC # 19321) were obtained from the American Type Tissue Collection (Rockville MD). All *Erwinia* strains were grown as overnight cultures in TSB (Tryptic Soy Broth-Difco) at room temperature. *Escherichia coli* TG-1 strain was obtained from Pharmacia Biotech (Laval PQ) and cultured in LB(Lennox L Broth -Difco) broth at 37°C with agitation.

5.1. Isolation of Erwinia DNA by DNAzol

The method provided by the manufacturers of DNAzol™ (Canadian Life Technologies) was used. Forty milliliter (40 ml) overnight cultures of bacterial cells grown to an OD_{600} of 0.5 were centrifuged and the supernatant discarded. One ml of DNAzol™ was added to the cell pellet and resuspended by pipette up and down gentle mixing. The sample was then allowed to stand at room temperature for 3 min. and the lysate transferred into two 0.5 ml aliquots. Ethanol (250 μL,

95%) was added; samples were incubated at RT for 5 minutes and microcentrifuged at maximum speed for 15 min in a microcentrifuge. The pellet was washed with 800 µl 95% ethanol twice and dried. DNA was solubilized by adding 1 ml distilled water and heating at 68°C for about one hour or less with intermittent mixing. Samples were sonicated by 10 second bursts to a maximum of 180 seconds to shear and reduce the DNA length. Some samples were further treated with RNase digestion, phenol/chloroform extraction and ethanol precipitation according to standard methods (Sambrook *et. al* . 1989). Samples were then spun, ethanol washed, dried and resuspended in distilled water.

5.2. Isolation of Erwinia DNA by Puregene ™

The method provided by the manufacturers of Puregene™ DNA Extraction Kit (Vector Biosystems) was used. A 5 ml overnight culture of *Erwinia* was centrifuged at 1000g for 5 minutes. Three milliliters (3 mL) of Cell Lysis Solution was added and the sample was incubated at 80°C for 5 minutes to lyse the cells. Fifteen µL of RNAse A Solution was added to the sample. The sample was inverted 25 times and incubated at 37°C for 15-60 minutes, then cooled. Protein Precipitation Solution (1 mL) was added and the sample vortexed at high speed for 20 seconds. The sample was centrifuged at 4800 rpm for 10 minutes. The supernatant was mixed with 3 ml of isopropanol by inversion 50 times. Sample was centrifuged at 2000g for 3 minutes, and the pellet was washed once with 3 ml 70% ethanol and air dried for 15 min. DNA Hydration Solution was added to give a concentration of 500 µg/ml, and DNA was rehydrated overnight (or alternately by heating at 65°C for one hour).

5.3. STMT Preparation of Crude DNA

An overnight culture of *Erwinia* (gram negative) bacteria was aliquoted into 1.5 ml aliquots in microfuge tubes and spun down at maximum rpm for 3 min. To each cell pellet was added 350 ul STMT Buffer (0.1 M NaCl, 10 mM Tris-Cl pH 8.0, 5% Triton-X100,10 mM $MgCl_2$) with optional reagents (1mg/ml lysozyme, 1 unit protein/µl lysostaphin, 10 mg/ml proteinase K, 100X [100 mg/ml] achromopeptidase) to resuspend the pellet. Then to each duplicate, a cocktail of 1 µl lysostaphin, 35 µl lysozyme and 3.5 µl of proteinase K were added, followed by 1.0 µl 100X achromopeptidase. All were allowed to lyse for five minutes. To end the lysis 15 µl of 0.5M EDTA was added. Samples were extracted once with phenol/chloroform, once with chloroform and EtOH precipitated. Samples were resuspended into their original volume and mixed with 8 µl of 0.5M EDTA. Samples were incubated at 37 °C for 15 minutes with 1 µl of 10 mg / ml RNase A. Ten microliter (10 µl) aliquots were then analyzed by (1% agarose) gel electrophoresis.

5.4. Sonication of genomic DNA

Highly viscous genomic DNA was sheared into predominantly 30Kb fragments by passing the bacterial DNA solution 10-20X's forcefully through a 22 gauge syringe needle. The process was then be repeated using a 26 gauge syringe needle. Alternately, highly viscous genomic DNA solutions was efficiently sonicated into DNA fragments < 30Kb in length using a Virsonic 50 sonicator (Virtis). Sonication was performed at 40-50% max.energy output with the DNA sample kept on ice. Ten second (10 sec) sonication bursts were performed with 10-15 second rest periods between bursts until the desired range of DNA fragments lengths (500bp to 3000bp) was generated (typical cummulative sonication time of 180 seconds for DNA solutions $\leq$ 1 mg/L).

6. Polyacrylamide Gel Electrophoresis

Gel electrophoresis of radiolabelled probes and isotopic CPT assay samples was carried out on denaturing 20% polyacrylamide/7M urea gels (Maniatis 1989) using a Bio-Rad Mini-Protean II apparatus. Minigels were prepared fresh before use and pre-run at 40 V/cm for 15 minutes in 1 x TBE buffer at room temperature. Sample wells were flushed and samples loaded and run at 40 V/cm for 1 hour in 1 x TBE buffer at room temperature.

7. Autoradiograms and Data Analysis

After running, gels were disassembled, placed on Parafilm and wrapped in plastic wrap. Wrapped gels were exposed overnight on Kodak XAR-5 film (Interscience) in X-ray cassettes with a phosphor screen at -20 °C. X-ray film was developed in Kodak GBX developer and fixer (2 minutes developer, 1 minute water rinse, 3 minutes fixer). For data analysis, autoradiograms were scanned using a HP ScanJetIIcx scanner (150x150 dpi) attached to a Macintosh computer. Densities of intact and cleaved probe bands were calculated from the autoradiograms, using the public-domain NIH Image 1.61 program for Macintosh. Densities were then translated into % cuts using the Microsoft Excel spreadsheet program. Samples were corrected for background by subtracting values for the negative control lanes from each band, and for variances in sample loading by normalizing values to the average of total pixels in all sample lanes. Using this method, samples varied by less than 10% in most cases. In cases where one gel could not contain all samples, at least one sample was run as a standard comparison for both gels and values corrected accordingly.

8. ^{32}P-CPT Assays with Synthetic Target

CPT assays with radiolablelled probe and synthetic target were performed in a total assay volume

of 10 µL. For basic assays, each sample contained 20 mM TES buffer pH 6.8, 0.05% Triton X-100, 4 mM $MgCl_2$, 0.01 µg/µL *Thermus thermophilus* RnaseH (ID Biomedical, Burnaby, BC), and 1 fmol of radiolabelled probe. Target concentrations ranged from 3 x 10^6 to 300 molecules per sample. Reactions were incubated at 60 °C for 30 minutes and stopped by addition of 10 µL of urea dye (8 M urea, 100 mM EDTA, 0.25% bromphenol blue). Samples were denatured at 95 °C for 5 minutes and flash-cooled prior to electrophoresis. Ten µL of each sample were separated on 20% polyacrylamide/7 M urea gels, and gels were exposed overnight to obtain autoradiograms. Samples to be analyzed for C2 activity included the above concentrations of reagents and probe, and used sterile distilled water in place of target. To confirm the presence of C2 activity, C2 samples were incubated in the presence of 10 mM EDTA.

9. ^{32}P-CPT Assays with Genomic Target

Assays with 3' ^{32}P-labelled probe were carried out as described for synthetic targets, with some modifications. EDTA (50 µM) and spermine (2 mM) were added to the cocktail mix to reduce background from non-specific probe-DNA interactions. Genomic targets (*Erwinia herbicola* and *E. coli* TG-1 genomic DNA) were sonicated to 0.5-2 kb fragments to improve reaction signals. Amounts of genomic target were calculated according to number of chromosomal copies (5 x 10^7 and 5 x 10^4 copies; 50 ng and 50 pg target per reaction respectively). Each sample was performed in triplicate. Finally, genomic targets were denatured at 94 °C for 5 minutes and flash-cooled prior to CPT assay.

10. CPT Assays/CE Detection

CPT assays for CE detection were carried out essentially as described for CPT assays with ^{32}P labelled probes with some modifications. In place of urea dye, 10 mM EDTA (fluorescein-labelled probe) or 50 mM EDTA (Cy5-labelled probe) was used to stop the reaction for some assays. Twenty µL samples were loaded into CE microvials for analysis. Assays varied as described in the Results section to optimize probe signals. Genomic samples were not denatured prior to assay, nor were samples denatured before CE detection. CPT assays were analyzed by capillary electrophoresis on 27 or 37 cm x 100 µm i.d. eCAPssDNA100 columns (Beckman) in tris/borate/7M urea buffer as described above for probe analysis. Samples were injected at 7.5 kV for 3 seconds (normal polarity mode; length to detector 7 cm; capillary temperature 30 °C). Separation voltages were adjusted to 300 V/cm depending on total column length. Probe cleavage was expressed as the percentage of the total peak area of cut and intact probe for each target

concentration. Probe cleavage values were corrected for C2 activity. Samples were performed in duplicate.

11. 3'-Biotin Labelling of EH Probes

5'-Fluoresceinated EH probes were 3'-end-labelled with biotin for CPT-CE studies. One hundred pmol of probe was labelled using TdT and biotin-16-ddUTP (SIGMA Chemical Co., St. Louis MO) as above for ^{32}P labelling, using a labelling ratio of 10 mol biotin-16-ddUTP : 1 mol 3'-OH ends. Labelled probes were purified on a NICK column (Pharmacia) and eluted in a volume of 400 μL distilled water (final concentration 250 nM). Aliquots of probes were analyzed by capillary gel electrophoresis for purity as described above.

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Appendix 1. R646 DARPA-CPT Contract Deliverables

1. Delivered to the University of Alberta:

1.1. April 30, 1998:

Deliverable	Lot #	Amount
Probes		
ERWTPL1224-28mer CPT probe, 5' fluorescein (EH8)	P-980401-3	5 x 20 µL @ 200 µM
Targets		
cEH8	S-980330-1	5 x 20 µL @ 20 µM
Er. herbicola DNA	G-971202-1	3 x 15 µL @ 0.5 µg/µL
Er. ananas DNA	G-971202-3	3 x 15 µL @ 0.5 µg/µL
Er. carotovora DNA	G-971202-2	3 x 15 µL @ 0.5 µg/µL
E. coli TG1 DNA	G-980430-1	3 x 15 µL @ 0.27 µg/µL

Appendix 1 (continued):

1.2. March 24, 1999:

Deliverable	**Lot #**	**Amount**
Probes		
ERWTPL1224-28mer CPT probe, 5' fluorescein (EH8)	P-980401-3	1 x 20 µL @ 200 µM
ERWTPL1224-28mer CPT probe, 5' fluorescein/3' biotin (EH8-B)	P-990311-1-6 P-990317-1-4	6 x 350 µL @ 250 nM 4 x 350 µL @ 250 nM
ERWTPL1224-28mer CPT probe, 5' Cy5/3' biotin (Cy5-EH8)	P-981026-1	6 x 20 µL @ 20 µM
Targets		
cEH8	S-990112-1	50 µL @ 121 µM
cEH8	S-990311-1	50 µL @ 86 µM
cEH8	S-980505-1	50 µL @ 153 µM
Er. herbicola DNA	91798	25 x 100 µL @ 0.1 µg/µL
Er. ananas DNA	91798	25 x 100 µL @ 0.1 µg/µL
Er. carotovora DNA	91798	25 x 100 µL @ 0.1 µg/µL
Er. uredovora DNA	91798	25 x 100 µL @ 0.1 µg/µL
E. coli TG1 DNA	91798	25 x 100 µL @ 0.1 µg/µL

Appendix 1 (continued):

2. Delivered to Mr. D. Bader, Scientific Authority, DRES:

2.1. Probes:

Supplied as described below. Store at -20 or -70 °C (longterm).

Deliverable	**Lot #**	**Amount/Description**
5' Fluorescein Labelled:		
EHBGL279-28 (EH1)	P-980312-1	17 x 20 µL @ 10 µM in water
EHBGL279-28 (EH1)	P-971204-1	11 x 20 µL @ 200 µM in TES[1]
EHBGL610-30 (EH2)	P-980312-2	17 x 20 µL @ 10 µM in water
EHBGL610-30 (EH2)	P-971204-2	10 x 20 µL @ 200 µM in TES
EHBGL1044-31 (EH3)	P-980312-3	15 x 20 µL @ 10 µM in water
EHBGL1044-31 (EH3)	P-971125-1	7 x 20 µL @ 200 µM in TES
EHBGL1044-31 (EH3-Hyb) (unlabelled)	P-971008-1	4 x 20 µL @ 119 µM in TES
EHBGL1468-31 (EH4)	P-980312-4	17 x 20 µL @ 10 µM in water
EHBGL1468-31 (EH4)	P-971204-3	10 x 20 µL @ 200 µM in TES
EHETZGENE858-30 (EH5)	P-980401-1	17 x 20 µL @ 200 µM in water
EHETZGENE1175-26 (EH6)	P-980401-2	17 x 20 µL @ 200 µM in water
ERWTPL1224-28 (EH8)	P-980401-3	6 x 20 µL @ 200 µM in water
ERWTPL1158-28 (EH10)	P-980401-4	26 x 20 µL @ 200 µM in water
ERWTPL1751-26 (EH11)	P-980401-5	32 x 20 µL @ 200 µM in water

5' Fluorescein/3' Biotin:

ERWTPL1224-28 (EH8-B)	P-990317-5-8	4 x 400 µL @ 250 nM in water

5' Cy5/3' Biotin:

ERWTPL1224-28 (Cy5-EH8)	P-981026-1	7 x 20 µL @ 20 µM in water

[1] TES = 20 mM TES buffer pH 6.8

Appendix 1 (continued):

2.2 Synthetic Targets:

Supplied in distilled water; store at -20 or -70 °C.

Deliverable	Lot #	Amount/Description
cEH1	S-971128-2	3 x 20 µL @ 200 µM
cEH2	S-971128-3	2 x 20 µL @ 111 µM
cEH3	S-971128-1	3 x 20 µL @ 180 µM
cEH4	S-971128-4	2 x 20 µL @ 164 µM
cEH5	S-980505-2	90 µL @ 131 µM
cEH6	S-980331-2	50 µL @ 206 µM
	S-980505-3	90 µL @ 122 µM
cEH8	S-980331-1	20 µL @ 213 µM
	S-980505-1	40 µL @ 153 µM
	S-990112-1	40 µL @ 121 µM
	S-990311-1	40 µL @ 86 µM
cEH10	S-980505-4	90 µL @ 93 µM
cEH11	S-980706-1	90 µL @ 143 µM

Appendix 1 (continued):

2.3. Genomic Targets:

Supplied in distilled water. Store at -20 or 4 °C.

Deliverable	**Lot #**	**Amount/Description:**
Er. ananas DNA	1103C (unsonicated)	8 x 100 µL @ 0.74 µg/µL [1]
	62598D	1 x 500 µL @ 0.58 µg/µL
	70798D	1 x 500 µL @ 0.62 µg/µL
	91798	180 x 100 µL @ 0.1 µg/µL
Er. carotovora DNA	1103B (unsonicated)	8 x 100 µL @ 0.73 µg/µL [1]
	62598B	1 x 500 µL @ 0.78 µg/µL
	70798B	1 x 500 µL @ 0.50 µg/µL
	91798	180 x 100 µL @ 0.1 µg/µL
Er. herbicola DNA	1103A (unsonicated)	8 x 100 µL @ 0.80 µg/µL [1]
	62598A	1 x 500 µL @ 0.78 µg/µL
	70798A	1 x 500 µL @ 0.50 µg/µL
	91798	180 x 100 µL @ 0.1 µg/µL
Er. uredovora DNA	1103D (unsonicated)	8 x 100 µL @ 0.16 µg/µL [1]
	62598C	1 x 500 µL @ 0.10 µg/µL
	70798C	1 x 500 µL @ 0.62 µg/µL
	91798	180 x 100 µL @ 0.1 µg/µL
E. coli TG-1 DNA	11126	8 x 400 µL @ 0.27 µg/µL [2]
	72598A	1 x 500 µL @ 0.15 µg/µL
	72598B	1 x 500 µL @ 0.14 µg/µL
	91798	180 x 100 µL @ 0.1 µg/µL

[1, 2] Delivered previously

Appendix 2. Sample Box Allocations for DARPA-CPT Deliverables to DRES

1. CPT Probes:

Box 1: EH1, EH2, EH3, EH3-Hyb, EH4 (200 μM in TES)

Box 2: EH1, EH2, EH3, EH4 (10 μM in water)

Box 3: EH5, EH6, EH8

Box 4: EH10, EH11

Box 5: Cy5-EH8, EH8-B

2. Targets:

Box 6: Synthetic target stocks: cEH1-cEH11

Box 7: Genomic target stocks: *Er. herbicola, Er. ananas*

Box 8: *Er. carotovora, Er.uredovora*

Box 9: *E. coli* TG1

Box 10-11: *Er. herbicola*

Box 12-13: *Er. ananas*

Box 14-15: *Er. carotovora*

Box 16-17: *Er.uredovora*

Box 18-19: *E. coli* TG1